# AN EXAMINATION OF THE ROLE OF THE CENTRAL OREXIN SYSTEM IN BINGE-LIKE ETHANOL CONSUMPTION

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## ABSTRACT

Jeffrey Jon Olney: An Examination of the Role of the Central Orexin System in Binge-Like Ethanol Consumption
(Under the direction of Todd Thiele)

Although centrally synthesized exclusively within the hypothalamus, orexin (OX) neurons project to various structures throughout the brain to modulate a host of physiological functions, including reward processing and stress responding, each of which contributes to the likelihood that an individual may drink ethanol. More recent evidence has also implicated this system in modulating the neurobiological responses to ethanol. The goal of the present dissertation was to further examine the role of the OX system in neurobiological responses to ethanol by characterizing the participation of this peptide system in binge-like ethanol drinking behavior. We assessed the impact of repeated episodes of binge-like ethanol drinking on different aspects of the OX system in Chapter 2 and found that binge-like ethanol drinking caused evidence of increased OX signaling, particularly within the lateral hypothalamus (LH), but did not produce lasting changes in mRNA expression. In Chapter 3, we used site-directed pharmacological tools to examine the individual contribution of each OX receptor, the OX1R and OX2R, within the ventral tegmental area (VTA) in binge-like ethanol drinking. Here, we observed that signaling onto the OX1R, but not OX2R, selectively modulates binge-like ethanol drinking without affecting sucrose consumption. Moreover, further investigations revealed that it does so independent of stress modulation as inhibiting intra-VTA OX1Rs did not alter anxiety-like behavior. Similarly, Chapter 4 was designed to characterize the participation of each OXR within the central nucleus of the amygdala (CeA). Using selective antagonists directly infused

into the CeA, we found that binge-like ethanol drinking within this region is predominately regulated by the OX1R and that this circuitry is independent of that which modulates responding to natural reinforcers and stress as inhibiting OX1Rs within the CeA did not affect binge-like sucrose consumption nor did it alter anxiety-like behavior. Together, these data indicate that the OX system significantly contributes to binge-like ethanol drinking and reveal that the LH→VTA and LH→CeA are two key pathways that selectively modulate this behavior. More broadly, these findings implicate the OX system as a highly promising target for the treatment of alcohol use disorders.

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| | |
|---|---|
| 3V | third ventricle |
| ACTH | adrenocorticotropic hormone |
| AP | anterior/posterior |
| AUD | alcohol use disorder |
| BEC | blood ethanol concentration |
| BLA | basolateral amygdala |
| C57 | C57BL/6J mouse |
| CeA | central nucleus of the amygdala |
| CIE | chronic intermittent ethanol |
| CPP | conditioned place preference |
| CRF | corticotropin-releasing factor |
| D3V | dorsal third ventricle |
| DA | dopamine |
| DAB | 3,3′-diamino-benzidine tetrahydrochloride |
| DID | drinking in the dark |
| DMH | dorsomedial hypothalamus |
| DMSO | dimethyl sulfoxide |
| DREADD | designer receptors exclusively activated by designer drugs |
| DV | dorsal/ventral |
| f | fornix |
| FSCV | fast scan cyclic voltammetry |
| HPA | hypothalamic-pituitary-adrenal |

| | |
|---|---|
| ic | internal capsule |
| IHC | immunohistochemistry |
| IR | immunoreactivity |
| KOR | kappa opioid receptor |
| LC | locus coeruleus |
| LH | lateral hypothalamus |
| LSD | least significant difference |
| ML | medial/lateral |
| mRNA | messenger RNA |
| mt | mammillothalamic tract |
| Nac | nucleus accumbens |
| NAcSh | shell of the nucleus accumbens |
| opt | optic tract |
| OX | orexin |
| OX1R | orexin-1 receptor |
| OX2R | orexin-2 receptor |
| OXR | orexin receptor |
| PBS | phosphate buffer saline |
| PCR | polymerase chain reaction |
| PFA | perifornical area of the hypothalamus |
| PL | prelimbic cortex |
| PVN | paraventricular nucleus of the hypothalamus |
| PVT | paraventricular nucleus of the thalamus |

| | |
|---|---|
| RT-PCR | real time polymerase chain reaction |
| SB | SB-334867 |
| sox | supraoptic decussation |
| TCS | TCS-OX2-29 |
| VTA | ventral tegmental area |

# CHAPTER 1

## GENERAL INTRODUCTION

Ethanol is produced via the biochemical process of fermentation when yeast metabolizes sugars (e.g. glucose: $C_6H_{12}O_6$) into ethanol and carbon dioxide ($C_2H_5OH + CO_2$). Early humans took advantage of this process to produce alcoholic beverages; in fact, it is believed that the earliest iteration was most likely mead, which is made from fermented honey, and is estimated to have emerged during the Paleolithic Age (8000 BCE; Ray and Ksir, 2002). Since then, alcohol has been a major part of human society and was even found referenced in Hammurabi's code (1720 BCE), which included a mandate to set the price and quality of beer (Mandelbaum, 1965). In fact, alcohol, having approximately 7 kcal per gram (The National Agricultural Library, 2015), was a major component of a nutrient-rich diet at the time (Homan, 2004). Additionally, the fermentation process eliminates most harmful bacteria present in the water supply (Vriesekoop *et al*, 2012) and was considered a source of sanitary hydration (Homan, 2004). For these reasons, early settlers of colonial America referred to alcohol as the "Good Creature of God," yet they were able to differentiate between drinking for hydration and nutrients versus drinking for intoxication- considering the latter to be a sinful act (Acker and Tracy, 2004).

This disdain for alcoholic inebriation would drive much of the public's opinion of alcohol consumption over the next several centuries. This attitude would reach fever pitch in the early 20$^{th}$ century and ultimately resulted in the 18$^{th}$ Amendment to the U.S. Constitution, which banned the production, storage, and consumption of alcoholic beverages. Interestingly, although

alcohol consumption dropped precipitously during the years of Prohibition (1919-1933), consumption was not fully eradicated (Nephew *et al*, 1999)- indicating that a portion of the American population opposed the enactment and chose to continue drinking despite the ban. Over the fourteen years of Prohibition, popular opinion steadily shifted and drinking rates continued to climb until the 21st Amendment overturned Prohibition (Levine, 1984; Nephew *et al*, 1999). Since then, alcohol has remained a mainstay of American culture. In fact, Americans spend over $100 billion each year purchasing alcoholic beverages (Levine, 1984), but the health and socioeconomic cost of alcohol use far exceeds the monetary value generated from its sales.

**Alcohol Use and Abuse**

Alcohol abuse can lead to a host of harmful health conditions (Castaneda *et al*, 1996; Rehm *et al*, 2003; Sanap and Chapman, 2003; Smith *et al*, 1999) and remains the third leading cause of preventable death in the United States (Mokdad *et al*, 2004)- claiming an estimated 90,000 lives each year (Naimi *et al*, 2003). In addition to adverse health consequences, alcohol is also associated with high economic costs. Indeed, the loss of work productivity, healthcare expenditures, and strain on the criminal justice system- among others- due to alcohol abuse has been estimated to cost nearly $250 billion (Sacks *et al*, 2015). These costs are likely driven by the widespread use of alcohol as epidemiological surveys have found that the vast majority (nearly 88%) of American adults have reported consuming alcohol at least once in their lifetime while 71% had reported drinking during the past year and nearly half the past month (SAMHSA, 2014). Moreover, it has been estimated that over 17 million Americans currently meet the criteria for at least one alcohol use disorder (AUD; SAMHSA 2012). In fact, the sheer magnitude of alcohol consumption is best illustrated by the fact that some investigators have

chosen to express the units of annual per-capita alcohol consumption in gallons (Nephew *et al*, 1999; U.S. Census Bureau, 2012).

*Binge Alcohol Drinking*

One of the major factors that contributes to the large volume of alcohol consumption and high prevalence of AUDs is binge alcohol drinking, which has recently been defined by the National Institute on Alcohol Abuse and Alcoholism as the consumption of enough alcohol over a short period to achieve a blood ethanol concentration (BEC) of at least 80 mg/dl (NIAAA Newsletter, 2004). In fact, of the nearly $250 billion annual cost attributed to alcohol abuse referenced above, a staggering $191.1 billion (76%) of that total is due to binge alcohol drinking (Sacks *et al*, 2015). Although young adults make up a significant proportion of individuals who engage in binge drinking, approximately 70% of binge drinking episodes involve adults over the age of 26 (Naimi *et al*, 2003). Indeed, nearly 25% of adults surveyed have reported to have recently engaged in binge drinking (SAMHSA, 2014). Moreover, binge drinking is an activity adults frequently partake in as one in six report binge drinking about four times each month (CDC, 2010). In fact, 75% of all alcohol consumed in the United States is done so in the form of a binge (OJJDP, 2005). This is particularly problematic as frequent binge drinking may lead to alcohol dependence and abuse (Knight *et al*, 2002; Rubinsky *et al*, 2010). Considering that approximately 1.5 million adults received treatment for an AUD in 2014 (SAMHSA, 2014), a better understanding of the neurobiological mechanisms that contribute to this harmful and costly disorder may be beneficial in facilitating the development of novel treatments for AUD's.

*Psychological Processes that Drive Alcohol Use and Abuse*

Despite the economic cost and adverse consequences to an individual's welfare, people are nonetheless driven to drink alcohol. Indeed, alcohol consumption is a complex human behavior; as such, the factors that motivate an individual to use or abuse alcohol are varied. It is known that social factors may play a part as peer relationships may drive alcohol drinking (Leung *et al*, 2014), yet for others chronic alcohol drinking may lead to more automatic, habitual patterns of alcohol drinking behavior devoid of enjoyment (Barker and Taylor, 2014; O'Tousa and Grahame, 2014). Although each of these factors deserves significant research attention in order to provide the most complete understanding of AUDs, the current dissertation will focus on two psychological processes: positive-reinforcement and negative-reinforcement. Positive-reinforcement can be defined as the presentation of a rewarding experience or stimulus that increases the probability the individual will engage in a behavior- in this case alcohol drinking. On the other hand, negative reinforcement is the relief or avoidance of a negative experience or stimulus, which increases the probability of alcohol drinking behavior.

The euphoric, rewarding aspects of alcohol are believed to be largely mediated by the recruitment of the mesolimbic dopamine (DA) system- although it should be noted that other neurotransmitter systems contribute to alcohol use and abuse and have been comprehensively reviewed elsewhere (Sommer and Spanagel, 2013). Indeed, ethanol has long been shown to increase extracellular levels of DA in the nucleus accumbens (NAc; Di Chiara and Imperato, 1988; Weiss *et al*, 1993). Consistent with this observation, blocking DA signaling has been shown to reduce ethanol seeking behavior (Czachowski *et al*, 2001; Hodge *et al*, 1997; Rassnick *et al*, 1992). Moreover, the positive-reinforcing properties of ethanol have been confirmed using a variety of behavioral paradigms. For example, ethanol has been shown to reduce intracranial

self-stimulation thresholds (Lewis and June, 1990) and induces conditioned place preference (CPP) in rodents (Bozarth, 1990). What is more, human imaging studies have revealed that alcohol and alcohol-related cues activate a number of regions within the reward system (Ingvar *et al*, 1998; Wrase *et al*, 2002) that leads to the release of DA within the ventral striatum (Boileau *et al*, 2003; Yoder *et al*, 2007). Together, these data indicate that ethanol possesses positive-reinforcing properties through its activation of the mesolimbic dopaminergic reward system.

It is generally believed that the initial stages of ethanol use are driven by the positive-reinforcing properties of the drug whereas negative-reinforcement becomes the more prominent motivator of ethanol abuse as neurobiological systems adapt to repeated ethanol challenges (Koob, 2003, 2013). However, negative reinforcement may also contribute to the early stages of ethanol use as well. In fact, numerous reports have described relatively high comorbidity rates of AUDs with stress/anxiety or major depression disorders (Arolt and Driessen, 1996; Kushner *et al*, 1990; Weissman *et al*, 1980). Although these high rates of comorbidity cannot indicate causation, studies involving nonhuman primates have demonstrated that stressful experiences lead to increased ethanol drinking (Barr *et al*, 2004; Higley *et al*, 1991). What is more, ethanol may help mitigate these psychologically unpleasant states. In addition to producing a euphoric effect (see above), which may help relieve symptoms of depression, ethanol has also been shown to have anxiolytic effects. Indeed, acute ethanol leads to a reduction in anxiety-like behavior in a number of different paradigms including open-field locomotor activity (Durcan and Lister, 1988) and the elevated plus maze (Pandey *et al*, 2008; Prunell *et al*, 1994). Further investigation suggests that the anxiolytic properties of ethanol are believed to be due, in part, to its actions in the CeA (Pandey *et al*, 2008). Together with the findings from the previous section regarding

the rewarding effects of ethanol, these data indicate that individuals may attempt to self-medicate with ethanol to achieve some semblance of relief from a negative affective state. Consistent with this hypothesis, individuals who drink for similar reasons (i.e. negative-reinforcement) have been found to be at a greater risk of developing AUDs (Ray *et al*, 2009).

**Modeling Excessive Ethanol Consumption in the Laboratory**

As outlined above, excessive alcohol consumption is a complex human disorder that affects a large proportion of the population. However, much of the research conducted in the laboratory uses animal models to investigate alcohol use, which is problematic as any one preclinical model is insufficient to address a multifaceted neuropsychological disorder such as AUDs. For example, two-bottle choice tests are well-suited to examine ethanol preference (Spanagel, 2000), which can then be used to identify genetic markers that may increase the risk of alcohol use (Belknap *et al*, 1997; Phillips *et al*, 1994). However, this procedure rarely yields physiologically relevant BECs, even in high-preferring strains of mice (Dole and Gentry, 1984), making it an unfit model to examine excessive ethanol consumption. Alternatively, procedures that induce reinstatement of ethanol seeking behavior are an ideal model for investigating the neurobiology of ethanol relapse (Spanagel and Hölter, 2000), but cannot fully address the defining characteristics of binge alcohol drinking (i.e. achieving a BEC of at least 80 mg/dl in a short period of time). Moreover, other techniques that model excessive ethanol consumption rely on experimenter delivered ethanol via injection, gavage, or vapor inhalation (Gilpin *et al*, 2008; Majchrowicz, 1975; Roberts *et al*, 2000) or may even require fluid deprivation (Cozzoli *et al*, 2009), the incorporation of ethanol in the animal's diet (Roy and Pandey, 2002), or weaning the animal on sweetened ethanol (Koob and Weiss, 1990). These methods introduce unnecessary confounds (e.g. stress, nutritional deficiencies, etc.) that could jeopardize

experimental interpretation. Importantly, the neuroadaptive consequences of drugs of abuse can vary depending how the drug was delivered (Jacobs *et al*, 2003). For these reasons, it is necessary that multiple models of AUDs be used to better capture the complexity of the human condition (Crabbe *et al*, 2011; Hines *et al*, 2005).

*Drinking in the Dark*

The "drinking in the dark" (DID) paradigm is a commonly used procedure to investigate binge-like ethanol intake in a preclinical model. This paradigm takes advantage of the nocturnal nature of rodents to promote high levels of consumption during the dark cycle that generates physiologically relevant blood ethanol concentrations (BECs) of 80 mg/dl or greater (Rhodes *et al*, 2005, 2007). Though other mouse lines can be used in this model, C57BL/6J, which are genetically predisposed to drink ethanol (Belknap *et al*, 1993; Rodgers, 1967; Rodgers and McClearn, 1962), have been found to be the optimal mouse line that exhibits the highest levels of ethanol consumption in this model (Rhodes *et al*, 2007). Importantly, further examination of this paradigm has indicated that DID-induced ethanol consumption does not stem from basic thirst (Rhodes *et al*, 2007) or caloric need (Lyons *et al*, 2008), which suggests that animals in this model are motivated to consume ethanol due to its post-ingestive effects rather than to maintain physiological homeostasis. Moreover animals readily drink ethanol in the DID procedure-making other techniques such as sucrose fading, food/water deprivation, or other time consuming and/or stress-inducing paradigms unnecessary. That said, it should be noted that mice that have experienced repeated binge cycles (up to 10 weeks) exhibited enhanced subsequent voluntary ethanol drinking, but did not show changes in anxiety-like behavior (Cox *et al*, 2013), which suggests that this procedure may not completely induce a transition to a dependence-like state in

the animals. Nonetheless, the DID procedure is a powerful tool that can be used to model aspects of human binge drinking.

The DID model also has the advantage of being a relatively simple procedure. On days 1-3 of the standard DID model, home-cage water bottles are removed three hours into the dark cycle and the animals are given access to test bottles containing ethanol (20% v/v) solution for two hours. Binge-like consumption is assessed on the fourth day, the test day, in which the procedures are similar to the previous days except that access to ethanol is often extended to four hours. After removing the ethanol bottles at the end of the four hour period on the test day, tail blood samples are collected from each animal in order to assess BECs.

**The Orexin System**

In 1998, two independent research groups simultaneously discovered the existence of a novel peptide. One group noted that this new peptide possessed structural similarities to the gut-peptide, secretin, but displayed an expression pattern limited to the hypothalamus (de Lecea *et al*, 1998); thus, these researchers deemed this new peptide hypocretin (hypothalamus + secretin = hypocretin). Alternatively, Sakurai and colleagues (1998) observed that the peptide was located in the hypothalamus, a brain region long-known for its critical involvement in feeding behavior (Brooks *et al*, 1946; Hetherington and Ranson, 1940). After demonstrating that this peptide produced a robust feeding response these researchers named this peptide orexin (OX), which is derived from the Greek *orexis* meaning appetite.

Cloning studies have revealed that the OX system is comprised of two peptides, orexin-A and orexin-B, which are derived from the precursor, prepro-orexin, and act on two G-protein coupled receptors, orexin-1 and orexin-2 receptors (OX1R and OX2R, respectively; de Lecea *et al*, 1998; Sakurai *et al*, 1998). These peptides interact with equal affinity at the OX2R, but

orexin-A has been found to have a greater affinity for OX1R with orexin-B exerting minimal actions on OX1R (Sakurai *et al*, 1998). Although neurons that synthesize OX peptide are restricted to the dorsal regions of the hypothalamus, orexinergic fibers project extensively throughout the brain and participate in a myriad of neurobiological functions. In fact, the origin and target location of these orexinergic connections can provide insight into the function of the OX system. For example, Sakurai and colleagues (1998) were able to shrewdly deduce its role in feeding behavior based solely on its limited expression within the hypothalamus. In addition to feeding behavior, the hypothalamus is also involved in maintaining arousal (Vanni-Mercier *et al*, 1984). It was suggested that the OX system also plays a role in sleep and arousal when it was observed that regions critically involved in this behavior- such as the tuberomammillary nucleus, raphe nuclei, and locus coeruleus- were densely innervated by OX neurons (Peyron *et al*, 1998). Shortly thereafter, it was demonstrated that proper OX functioning was necessary to maintain healthy waking states (Chemelli *et al*, 1999; Lin *et al*, 1999). Additionally, Peyron and colleagues (1998) also observed that OX neurons project the ventral tegmental area (VTA), the nucleus accumbens (NAc), amygdala, and bed nucleus of the stria terminals, which are regions known to be involved in reward and/or stress processing. Consistent with these projection patterns, it was later demonstrated that OX also modulates the positive reinforcing effects of rewards (Harris *et al*, 2005) as well as stress responding (Kuru *et al*, 2000).

What is more, it has been proposed that hypothalamic OX can be dichotomized based on distinct functional attributes. Specifically, lateral hypothalamic (LH) OX neurons have been postulated to be more involved in feeding and reward-related behaviors (Harris *et al*, 2005) while OX neurons from the perifornical area of the hypothalamus (PFA) and dorsomedial hypothalamus (DMH) are believed to be more associated with stress and arousal (Harris and

Aston-Jones, 2006). Moreover, this dichotomy generalizes to the receptors as well; OX1R is more associated with reward processing (Smith *et al*, 2009) and OX2R with wakefulness and arousal (Akanmu and Honda, 2005; Willie *et al*, 2003). However, more recent findings indicate that such a clear segregation of OX function based simply on hypothalamic origin may not be as apparent as originally believed (González *et al*, 2012).

*Orexin and Reward*

The hypothalamus has been known for well over fifty years to be integrally involved in reward-related behaviors (Olds, 1958; Olds and Milner, 1954). Consistent with these findings, hypothalamic OX projections facilitate the activity of several brain regions in the reward circuit, such as the VTA (Korotkova *et al*, 2003; Moorman and Aston-Jones, 2010; Vittoz *et al*, 2008) and shell of the NAc (NAcSh; Mori *et al*, 2010; Mukai *et al*, 2009). It is believed that OX mediates the positive-reinforcing effects of rewarding stimuli by modulating activity of VTA DA neurons. In fact, both orexin-A (Borgland *et al*, 2006) and orexin-B (Borgland *et al*, 2008) have been found to strengthen glutamatergic transmission onto VTA DA neurons that serve to enhance neuronal excitability within the VTA. Furthermore, treatment with OX peptide into the VTA (Korotkova *et al*, 2003; Narita, 2006) and NAcSh (Patyal *et al*, 2012) leads to elevated mesolimbic DA release. Moreover, central infusions of OX have been found to be reinforcing in a DA-dependent manner as local infusions of orexin-A into the VTA produced conditioned place preference (CPP), which was blocked by concomitant infusion of a DA receptor antagonist directly into the NAc (Taslimi *et al*, 2012). These findings and others provide a theoretical framework to aid in the understanding of how OX may modulate the neurobiological responses to various drugs of abuse (see Mahler et al. 2012 for review).

*Orexin and Stress*

Early researchers investigating the neurobiological mechanisms of the stress-response system recognized the relationship between adrenocorticotrophic hormone (ACTH) release from the pituitary gland and the subsequent release of corticosterone in response to a stressor (Sayers and Sayers, 1949); however, the role of the hypothalamus in this stress-response system was relatively poorly understood for several years to follow (Katsuki *et al*, 1955). Since that time, the hypothalamus has been firmly established as a critical modulator of stress and anxiety- a fact perhaps best illustrated by its inclusion in the name of the predominate stress system, the hypothalamic-pituitary-adrenal (HPA) axis. In accordance with its restricted expression within the hypothalamus, the OX system has been found to regulate stress responding.

Intracerebroventricular (i.c.v.) infusion of orexin-A has been shown to activate the paraventricular nucleus of the hypothalamus (PVN; Kuru *et al*, 2000)- the subregion of the hypothalamus that expresses corticotropin releasing factor (CRF) that initiates a stress response from the HPA axis. Not surprisingly, this group went on to demonstrate that similar i.c.v. infusion of OX leads to an increase in circulating ACTH and corticosterone levels. Moreover, hypothalamic OX neurons are activated in response to an environmental stressor (Sakamoto *et al*, 2004; Winsky-Sommerer *et al*, 2004). Indeed, OX neurons have been shown to possess reciprocal connections with CRF neurons (Winsky-Sommerer *et al*, 2004) and OX is capable of activating CRF neurons of the HPA axis as well as extra-HPA axis CRF neurons within the central nucleus of the amygdala (CeA; Sakamoto *et al*, 2004). In humans, OX levels have been found to be directly correlated with increased symptoms of anxiety (Johnson *et al*, 2010). Together, these data indicate that OX participates in stress and anxiety, in part, through its

connections with the CRF system- although the OX system also does so through other neurobiological means beyond just the CRF system (see Johnson *et al*, 2012 for review).

**Ethanol Engages the Orexin System**

Multiple studies have reported that experience with ethanol recruits the OX system. This relationship was first observed by Lawrence and colleagues (2006) when they reported that chronic ethanol drinking increased OX mRNA in the LH relative to ethanol naïve rats. Interestingly, Morganstern and colleagues (2010) reported that chronic ethanol consumption caused a significant reduction in hypothalamic OX mRNA while acute ethanol exposure significantly enhanced OX mRNA and orexin-A levels. What is more, Barson and colleagues (2014) recently assessed changes in OX mRNA following ethanol drinking in an intermittent-access paradigm and found increased OX expression in the hypothalamus. A similar pattern of effects has also been observed in ethanol seeking behavior. Indeed, context-induced renewal of ethanol seeking behavior was found to activate OX neurons in the DMH and LH, but not PFA while ethanol preference and seeking in the homecage was positively correlated with OX activity in the LH and PFA, but not DMH (Moorman *et al*, 2016). The literature investigating changes in OXRs in response to ethanol drinking is relatively sparse; however, increases in OX2R levels in the anterior paraventricular nucleus of the thalamus were observed following ethanol drinking (Barson *et al*, 2014). Similarly, a strong, positive correlation has been observed between ethanol consumption and hypothalamic OX1R expression- though this effect did not reach significance (Pickering *et al*, 2007). As a whole, a pattern emerges from this collection of findings that suggests that ethanol exposure results in an upregulated OX system. Whether or not binge-like ethanol consumption similarly perturbs the OX system, however, has yet to be determined.

Given the need to approach AUDs from multiple different perspectives (i.e. models), further examination of how this peptide system responds to binge-like ethanol drinking is necessary.

**The Orexin System Modulates the Neurobiological Responses to Ethanol**

Findings outlined in the previous section suggest that ethanol drinking is associated with elevated activity in the OX system; thus, it stands to reason that pharmacological manipulations of OX signaling would impact ethanol intake. Indeed, Lawrence and colleagues (2006) confirmed such an effect when they demonstrated that a systemic injection of the selective orexin-1 receptor antagonist, SB-334867 (SB), reduced operant self-administration of ethanol. Similarly, SB was found to reduce ethanol consumption and preference in high ethanol preferring rats (Moorman and Aston-Jones, 2009). Further investigations suggest that modulation of OX signaling via SB may be specific to ethanol as Jupp and colleagues (2011) found that SB significantly attenuated the motivational effects of ethanol but not sucrose. Although these data suggest an essential role for OX1R signaling in ethanol drinking, recent evidence suggests the OX2R is also capable of modulating responses to ethanol (Anderson *et al*, 2014; Barson *et al*, 2014; Shoblock *et al*, 2011). Moreover, more comprehensive investigations of the role of OX in ethanol drinking have revealed that signaling within reward-related structures is responsible for modulating this behavior. Indeed, direct infusions of orexin-A in the LH or PVN increased ethanol drinking (Schneider *et al*, 2007) while a non-selective OXR antagonist into the VTA significantly decreased ethanol self-administration (Srinivasan *et al*, 2012). As a whole, these findings indicate ethanol drinking directly parallels OX signaling in the reward pathway.

Recent evidence suggests that OX may mediate ethanol responding by both modulating its reinforcing properties as well as recruiting stress-related circuitry. Indeed, systemic antagonism of the OX2R blocks ethanol-induced CPP (Shoblock *et al*, 2011) and suppression of

systemic OXR signaling reduces breakpoints for ethanol responding in a progressive ratio schedule of reinforcement (Anderson *et al*, 2014; Jupp *et al*, 2011a). Additionally, SB blocks yohimbine-induced renewal of ethanol seeking behavior (Richards *et al*, 2008)- a procedure that engages stress-related systems (Funk *et al*, 2006; Vythilingam *et al*, 2000) and is thought to model stress-induced relapse of drug seeking behavior (Feltenstein and See, 2006; Lee *et al*, 2004). Beyond preclinical studies, OX levels have been found to be directly correlated with severity of alcohol withdrawal symptoms in humans (Bayerlein *et al*, 2011; von der Goltz *et al*, 2011). Together, these data support the notion that OX may regulate ethanol responding through both positive- and negative-reinforcement.

Despite these findings, the role of the OX system in binge-like drinking remains relatively unexplored. Considering recent evidence from our lab indicates binge-like ethanol drinking recruits different neurocircuitry relative to moderate-level ethanol consumption (Lowery *et al*, 2010; Lowery-Gionta *et al*, 2012; Sparta *et al*, 2008), examination of the participation of this peptide system in binge-like ethanol drinking is necessary. To date, we and others have demonstrated that systemic inhibition of signaling onto OX1Rs (Olney *et al*, 2015) and/or OX2Rs (Anderson *et al*, 2014) disrupts binge-like ethanol drinking without impacting general locomotor behavior. However, this effect was not specific to ethanol as these studies also showed that OXR antagonists similarly reduced binge-like sucrose and saccharin consumption, which suggests that OX may modulate binge-like consumption of general, salient reinforcer regardless of nutritional content. Taken together, these data clearly demonstrate that OX is involved in binge-like ethanol drinking behavior; however, further investigation is required in order to identify the brain regions that specifically modulate this effect.

**Goals of the Current Dissertation**

Broadly, the primary goal of the current dissertation is to provide a detailed characterization of the OX system in binge-like ethanol drinking behavior. This will be achieved using two main strategies: describing the effect of binge-like ethanol drinking on the OX system and characterizing the specific OX neurocircuitry involved in this behavior. The second chapter will describe how repeated episodes of binge-like ethanol drinking impacts different facets of the OX system. Here, we will examine changes in orexin-A and -B in hypothalamic subregions via immunohistochemistry. Additionally, we will use polymerase chain reaction to examine alterations in the expression of prepro-orexin mRNA within the hypothalamus and attempt to identify changes in OX1R and OX2R mRNA expression in the hypothalamus as well as OX projection regions relevant to ethanol drinking: the VTA and amygdala. The next two chapters will investigate the contribution of each OXR subtype within brain regions involved in reward- and stress-related processing. The third chapter will characterize the OX neurocircuitry within the VTA while the fourth will focus on that of the CeA. Additionally, these chapters will elucidate the overarching psychological process that modulates this behavior by also investigating whether OXR signaling in the VTA and CeA influences anxiety-like behavior. Findings obtained from this series of experiments will provide valuable insight into the neurobiological mechanisms that underlie binge-like ethanol drinking behavior and will greatly expand our current understanding of the contribution of the OX system in binge-like ethanol drinking behavior. Importantly, these findings may greatly inform the development of pharmacological interventions to be used to treat AUDs and may reveal the OX system as a potential target for such treatment options.

# CHAPTER 2

## THE EFFECTS OF BINGE-LIKE ETHANOL CONSUMPTION ON THE OREXIN SYSTEM

### Introduction

The two peptides of the orexin (OX) system, orexin-A and orexin-B, are 33- and 28-amino acid sequences, respectively, that are cleaved from the precursor, prepro-orexin (de Lecea *et al*, 1998; Sakurai *et al*, 1998). Neurons that produce these peptides are restricted to the hypothalamus; however, these neurons send widespread projections to a number of regions throughout the brain. OX peptides from these fibers act on two receptors, the orexin-1 (OX1R) and orexin-2 receptor (OX2R), to influence a wide array of neurobiological functions including feeding (Sakurai *et al*, 1998), sleep (Chemelli *et al*, 1999), stress (Kuru *et al*, 2000), and reward (Harris *et al*, 2005). Interestingly, although there is a high degree of overlap in expression between the OXRs, the two subtypes display differential expression in some brain areas. For example, it has been reported that both receptors are moderately expressed in the ventral tegmental area (VTA) yet the OX1R is more densely expressed in the extended amygdala as well as the prelimbic and infralimbic cortices while the OX2R is more robustly expressed throughout much of the hypothalamus and the lateral habenula in the rat brain (Marcus *et al*, 2001).

These findings suggest a degree of separation in how each subtype participates in different functions. Indeed, the OX system has been shown to modulate the sensitivity of other

homeostatic regulators with the OX1R controlling insulin sensitivity (Shiuchi *et al*, 2009) while the OX2R controls leptin sensitivity (Funato *et al*, 2009). Perhaps the most notable distinction is regarding the involvement in reward and arousal, which are modulated by the OX1R and OX2R, respectively (Dugovic *et al*, 2009; Gozzi *et al*, 2011; Harris *et al*, 2005; Lin *et al*, 1999; Malherbe *et al*, 2009; Smith *et al*, 2009). Considering that neurons originating from the lateral hypothalamus (LH) have been reported to be more involved in reward while those of the dorsomedial and perifornical regions (DMH and PFA, respectively) predominately contribute to arousal and stress (Harris and Aston-Jones, 2006), these functional differences may arise from differences in anatomical sources of orexinergic fibers as well.

Moreover, both OXRs have been implicated in ethanol drinking (Anderson *et al*, 2014; Srinivasan *et al*, 2012), although disparities still exist in how each subtype contributes to neurobiological responses to ethanol. For example, Barson and colleagues (2014) demonstrated that the OX2R, but not OX1R, within the anterior paraventricular nucleus of the thalamus (aPVT) modulates ethanol consumption. Similarly, Shoblock and colleagues (2011) showed that treatment with an OX2R antagonist blocked ethanol-induced conditioned place preference- an effect that was not observed following OX1R inhibition. Together, these data and others indicate that the two OXR subtypes may be differentially involved in the neurobiological responses to ethanol.

What is more, multiple sources have reported observing ethanol-induced changes in the OX system. Specifically, a rather consistent pattern of effects has emerged that suggests that exposure to ethanol causes subsequent increases in OX levels (Lawrence *et al*, 2006; Morganstern *et al*, 2010). Relatively few studies have investigated relative differences in OX expression between the two peptides and/or receptors. However, what little evidence exists

suggests different aspects of the OX system undergo changes following experience with ethanol. Indeed, the aPVT shows elevated OX2R, but not OX1R, mRNA expression after ethanol exposure (Barson *et al*, 2015). Furthermore, this same study also reported no such changes in the posterior PVT, which suggests that changes in the OX system are not only specific to certain aspects of the OX system (e.g. receptor subtype) but also to particular regions of the brain.

As it stands, the current literature suggests that ethanol exposure leads to plastic changes in the OX system. However, the perturbations of the OX system following binge-like ethanol drinking behavior remain relatively unknown. Therefore, the purpose of the present study was to characterize the changes in the OX system following binge-like ethanol drinking using the "drinking in the dark" (DID) paradigm. These changes were assessed using real-time polymerase chain reaction (RT-PCR) to measure mRNA expression of prepro-orexin and each OXR subtype in the hypothalamus as well as the ventral tegmental area (VTA) and amygdala- two regions that receive dense orexinergic projections and are involved in ethanol drinking (Engel and Jerlhag, 2014; Gilpin *et al*, 2015; Peyron *et al*, 1998). To further characterize the effect of binge-like ethanol drinking on the OX system, levels of orexin-A and orexin-B were directly assessed using immunohistochemistry (IHC) within the LH, PFA, and DMH.

## Methods

### *Animals*

Male C57BL/6J mice (C57; Jackson Laboratories, Bar Harbor, ME), aged 6-7 weeks and weighing 20-25 g upon arrival, were used in each of the following studies. Mice were individually housed in plastic cages located in a vivarium with an ambient temperature of approximately 22ºC and a reverse light/dark cycle with lights off at 8:30 am. All animals had *ad*

*libitum* access to food and water except when specified below. All procedures used were in accordance with the National Institute of Health guidelines and were approved by the University of North Carolina Institutional Animal Care and Use Committee.

*Drinking in the Dark*

Three groups of fifty C57 mice experienced one or three cycles of "drinking-in-the-dark" (DID) in order to assess changes in the OX system following binge-like drinking behavior using immunohistochemistry (IHC) and RT-PCR. One cohort each was used for the orexin-A IHC, orexin-B IHC, and RT-PCR experiments. The DID procedure is a commonly used animal model of binge-like ethanol drinking that promotes high levels of consumption and generates physiologically relevant blood ethanol concentrations (BECs) of 80 mg/dl or greater (Rhodes *et al*, 2005, 2007). On days 1-3, standard water bottles were removed three hours into the dark cycle and the animals were given access to test bottles containing water, ethanol (20% v/v), or sucrose (3% w/v) solutions for two hours. Binge-like consumption was assessed on the fourth day when access to the test bottles was extended to four hours. Each four day testing period constituted a binge cycle, and there were 3 days with no ethanol access between binge cycles. The start dates of the binging cycles were staggered such that all groups finished testing on the same day and at the approximate same age. Moreover, all animals were sacrificed and perfused immediately following the final binge session in order to assess changes in the OX system while ethanol was still present in the brain.

*Immunohistochemical Analysis*

On the final day of testing, tail-bloods were collected and analyzed using the Analox blood ethanol analyzer (Analox Instruments, Luneburg, MA) to measure BEC. To ensure consistency across all groups, tail-bloods were collected from all animals; however, only those

blood samples from animals in the one and three cycle ethanol groups were analyzed. Animals were then sacrificed and transcardially perfused with 0.1 mM phosphate-buffered saline (PBS; pH7.4) followed by 4% paraformaldehyde in phosphate buffer. Brains were then collected and post-fixed in paraformaldehyde for 48 hr at 4°C, at which point they were transferred to PBS. The brains were cut using a vibratome (Leica Microsystems, Buffalo Grove, IL) into 40 μm sections that were stored in cryopreserve until the IHC assay. Sections were then transferred to PBS for 24 hr before processing with orexin-A or orexin-B antibodies. After rinsing in fresh PBS 4 times (10 minutes each), tissue sections were blocked in 10% goat serum and 0.1% triton-X-100 in PBS for 1 hour. Sections were then transferred to fresh PBS containing primary antibody for 72 hr at 5°C. Protein expression was detected using primary antibody against orexin-A (1:5000, Santa Cruz Biotechnology, Dallas, TX) or orexin-B (1:5000, Santa Cruz Biotechnology, Dallas, TX). After the 72 hr of incubation with antibody, the sections were rinsed four times and then processed with Vectastain Elite kits (Vector Labs, Burlingame, CA). The sections processed for orexin-A and -B were visualized by reacting the sections with a 3,3′-diamino-benzidine tetrahydrochloride (DAB; Polysciences, Inc., Warrington, PA) reaction solution containing 0.05% DAB, 0.005% cobalt, 0.007% nickel ammonium sulfate, and 0.006% hydrogen peroxide.

Digital images of peptide immunoreactivity (IR) were then obtained on a Nikon E400 microscope equipped with a Nikon Digital Sight DS-U1 digital camera run with Nikon-provided software. Immunoreactive neurons were counted using Image-Pro Plus software (MediaCybernetics, Rockville, MD). Anatomically matched pictures of the left and right sides of the brain were used to produce an average cell count for each hypothalamic subregion (LH, PFA, and DMH) of each animal. Only one section per side was analyzed in the current study.

The section for each animal was selected as the section with the most robust OX expression observed in the hypothalamus, which was approximately 1.06 to 1.34 mm posterior to bregma. The entire 40 µm section was examined. We adopted the boundaries set forth by Mahler and Aston-Jones (2012) for the current experiment (Fig. 2.1). Specifically, cells were qualified as LH OX cells if they were in the lateral portions of the hypothalamus between the internal capsule and the lateral edge of the fornix. Neurons found within the lateral edge of the fornix to the lateral edge of the mammillothalamic tract were considered cells of the PFA. Finally, neurons in the most medial portions of the hypothalamus between the lateral edge of the mammillothalamic tract and the third ventricle were recorded as DMH cells. However, upon initial quantification of the levels of orexin-A positive cells in our animals, it became clear that the DMH exhibited minimal orexin-A IR in our samples (grand mean: 3.327, SEM: 0.352); therefore, this subregion was removed from further analysis of both orexin-A and orexin-B peptides.

*Real Time Polymerase Chain Rection*

On the final day of DID testing, brains were immediately extracted via rapid decapitation and flash frozen using isopentane and stored at -80°C. Blood samples were collected immediately following rapid decapitation for the one and three cycle ethanol groups in order to assess BECs as described above. Bilateral cylindrical punches (1 mm x 1mm) containing target brain structures were collected from each brain and immediately submerged in RNALater (Ambio; Carlsbad, CA) per the manufacturer's instructions. Punches containing the hypothalamus and amygdala were taken starting at 1.70mm posterior to bregma while punches containing the VTA were taken 3.08mm posterior to bregma. RT-PCR analysis of mRNA expression was performed by the UNC Animal Clinical Chemistry and Gene Expression Laboratories and has been described previously (Kim *et al*, 2002). The nucleotide sequences for

the PCR primers and fluorogenic probes used in the analysis were as follows: prepro-orexin forward: 5'-AGG CCT CCA GGG ACG GCT-3', reverse: 5'-CAT GGT CAG GAT GCC AGC T-3', probe: 5'-FAG CGC CTC CTT CAG GCC AAC GGT A Q-3'; OX1R forward: 5'-CTT CTC CCA CTG GCT AGT GT-3', reverse: 5'-TGC TCC CGG AAT TTG CCA CT-3', probe: 5'-FTG CCG CCA ACC CTA TCA TCT ACA AC Q-3'; OX2R forward: 5'-CTC ACC AGC ATA AGC ACA CT-3', reverse: 5'-TGG TAC TCC CTG CTG TAG AT-3', probe: 5'-FTG AAG CGG TCC TGC CCC GTT GGC Q-3'; β-actin forward: 5'-CTG CCT GAC GGC CAG GTC-3', reverse: 5'-CAA GAA GGA AGG CTG GAA AAG A-3', probe: 5'-FCA CTA TTG GCA ACG AGC GGT TCC GQ-3'. Measurements were normalized to the water control group and presented as percent change from controls.

*Data Analysis*

Separate univariate ANOVAs were used to measure either orexin-A or orexin-B positive cells in either the LH or PFA using group (water, one-cycle ethanol, one-cycle sucrose, three-cycle ethanol, or three-cycle sucrose) as the independent variable. Similarly, separate univariate ANOVAs were also used to measure prepro-orexin, OX1R, or OX2R expression in the hypothalamus, amygdala, or VTA using group (water, one-cycle ethanol, one-cycle sucrose, three-cycle ethanol, or three-cycle sucrose) as the independent variable. The brain tissue from an animal in the three-cycle ethanol group was damaged during the orexin-A staining process making it unable to be quantified and was removed from the analysis. To ensure that hypothalamic OX expression was not affected by differential drinking levels, separate *t*-tests were used to assess consumption of each test solution (i.e. ethanol or sucrose) on the final day of testing between animals in the one or three DID cycle groups. Similarly, a *t*-test was also performed on the BEC data to measure differences in ethanol metabolism as a function of DID

cycles (one or three). Finally, separate univariate ANOVAs were used to analyze changes in body mass (g) as a function of group. Tukey's LSD *post-hoc* tests and Bonferroni corrections were used when applicable.

**Results**

Animals' responses to the test solutions were comparable regardless of DID history as ethanol and sucrose drinking on the final day of testing did not differ between animals that experienced one- or three-cycles of DID (Figure 2.2A,C; $t_{(18)} = 0.1316$, $p = 0.897$; $t_{(18)} = 0.4746$, $p = 0.641$; respectively). Additionally, both groups of ethanol drinkers achieved similar BECs regardless of DID history (Figure 2.2B; $t_{(18)} = 0.603$, $p = 0.5540$). Moreover, no differences across any of the groups were observed in body mass as measured at the end of testing (Figure 2.2D; $F_{(4, 45)} = 0.541$, $p = 0.706$).

Our analysis revealed that binge-like consumption of ethanol or sucrose solutions significantly altered the number of orexin-A positive neurons within the LH (Figure 2.3A-F; $F_{(4,48)} = 5.863$, $p = 0.001$). Further probing of this effect revealed that relative to the water group (Figure 2.3B) all test groups (i.e. one-cycle ethanol (Figure 2.3C), three-cycle ethanol (Figure 2.3D)**,** one-cycle sucrose (Figure 2.3E), and three-cycle sucrose (Figure 2.3F)) displayed significantly reduced OX levels (Fig. 1A; $p$'s $< 0.010$). Similarly, binge-like consumption of ethanol or sucrose also significantly altered OX levels within the PFA ($F_{(4,48)} = 3.702$, $p = 0.011$). Unlike the LH, however, Tukey's LSD tests indicated that only the three-cycle ethanol group showed significant reductions in OX expression in the PFA relative to the water group ($p = 0.006$).

Our second cohort of animals used to investigate orexin-B IR also displayed comparable levels of binge-like ethanol (Figure 2.4A; $t_{(15)} = 0.697$, $p = 0.497$) and sucrose (Figure 2.4C; $t_{(17)}$

= 0.622, $p$ = 0.542) consumption as well as BECs (Figure 2.4B;$t_{(15)}$ = 0.480, $p$ = 0.638) regardless of the number of DID cycles. Consistent with the previous cohort, no differences were observed in body mass as a function of group (Figure 2.4D; $F_{(4, 47)}$ = 0.338, $p$ = 0.851).

As with the last analysis, we observed that binge-like consumption significantly impacted levels of orexin-B in the LH and PFA (Figure 2.5A-F; $F_{(4,39)}$ = 4.410, $p$ = 0.005; $F_{(4,39)}$ = 4.891, $p$ = 0.003; respectively). Further probing of the effect within the LH revealed that the one-cycle ethanol group (Figure 2.4C) displayed significantly reduced orexin-B levels relative to water drinking controls (Figure 2.4B; $p$ = 0.005) as well as relative to the three-cycle ethanol group (Figure 2.4D; $p$ = 0.005). Within the PFA, *post-hoc* analyses revealed that both the one-cycle ethanol and one-cycle sucrose groups exhibited orexin-B levels that were significantly lower than the water drinking controls ($p$ = 0.002 and $p$ = 0.001, respectively).

The group of animals used in the PCR study displayed statistically similar levels of binge-like ethanol (Figure 2.6A; $t_{(18)}$ = -0.935, $p$ = 0.362) and sucrose (Figure 2.6C; $t_{(18)}$ = -0.795, $p$ = 0.437) consumption as well as BECs (Figure 2.6B; $t_{(18)}$ = 1.468, $p$ = 0.159) regardless of the number of DID cycles. The body mass of the animals did not vary as a function of group (Figure 2.6D; $F_{(4, 45)}$ = 0.828, $p$ = 0.515).

We detected a significant difference in prepro-orexin expression within the hypothalamus as a function of group (Figure 2.7A; $F_{(4, 45)}$ = 5.558, $p$ = 0.001). Here, the three-cycle sucrose group was found to have significantly great precursor mRNA relative to all of the other groups except the one-cycle ethanol group ($p$'s $\leq$ 0.002). We did not observe any significant changes in OX1R mRNA expression as a function of group in the hypothalamus (Figure 2.7B; $F_{(4, 45)}$ = 0.712, $p$ = 0.588) or VTA ($F_{(4, 45)}$ = 0.339, $p$ = 0.850). However, we did observe significant alterations in OX1R expression within the amygdala ($F_{(4, 45)}$ = 5.688, $p$ = 0.001), which was

driven by the fact that the three-cycle ethanol group displayed elevated OX1R mRNA relative to the water group ($p < 0.001$) and one-cycle sucrose group ($p = 0.003$). Levels of OX2R mRNA were below detectable levels in the amygdala and VTA; thus, we were unable to analyze those results. However, there was sufficient OX2R expression within the hypothalamus, but no differences were observed in this region (Figure 2.7C; $F_{(4, 43)} = 0.727$, $p = 0.578$).

**Discussion**

It has previously been reported that experience with ethanol engages the OX system and causes changes to peptide and/or receptor levels (Barson *et al*, 2015; Lawrence *et al*, 2006; Morganstern *et al*, 2010). Findings from the current studies expand upon our current understanding by demonstrating that binge-like ethanol drinking similarly alters the OX system. Moreover, further examination indicates that these changes are not due to differences in ethanol consumption, BECs, or body mass but rather are due to exposure to ethanol.

Our first experiment revealed that the levels of hypothalamic orexin-A are reduced following repeated cycles of binge-like ethanol drinking. Moreover, this effect was not specific for ethanol as a similar pattern of results was observed among animals that experienced binge-like sucrose consumption. However, these effects were specific to the LH, a subregion hypothesized to be mainly involved in reward processing (Harris *et al*, 2005), as we observed minimal changes in the PFA, a subregion believed to be more involved in stress and arousal (Harris and Aston-Jones, 2006). Although we observed an increase in orexin-A IR in the PFA among animals that experience three cycles of binge-like ethanol consumption, this effect may be due to ethanol's influence on normal sleep patterns (Ebrahim *et al*, 2013). Indeed, ethanol has been shown to promote sleep by inhibiting OX neurons in the PFA (Sharma *et al*, 2014).

Our orexin-A IR data appears to provide support for the dichotomy of OX function between the LH and PFA in processing reward and arousal/stress, respectively (Harris and Aston-Jones, 2006). Our findings of the changes in orexin-B, on the other hand, do not support this theory. Similar to orexin-A, we observed that levels of orexin-B decline following binge-like ethanol and sucrose. Unlike the other peptide, however, this reduction in orexin-B was observed in both the LH and PFA- indicating an absence of functional dichotomy. In fact, there are several other examples in the literature that do not support the idea of a clear separation of OX function between the LH and PFA. Most notably, González and colleagues (2012) infused retrograde tracers directly into the locus coeruleus (LC) and VTA- two regions that receive dense orexinergic input that are critically involved in sleep and reward, respectively- and found that OX neurons within the LH and PFA each projected substantially to both the LC and VTA. In fact, the authors even reported that OX neurons within the LH were more likely to project to the LC than the VTA. As a whole, these data indicate that the functional roles of OX cannot be so clearly segregated based on hypothalamic origin.

Contrary to our findings with orexin-A, we also observed that the decline of orexin-B positive neurons was transient. Specifically, we observed a reduction in orexin-B IR following one-cycle of binge-like consumption of a salient reinforcer; however, levels returned back to baseline for the animals that experienced three-cycles of DID. This suggests that orexin-B is initially engaged during the early stages of DID but is no longer recruited with repeated use. In terms of alcohol-use disorders, this may suggest that orexin-B is involved in the initial stages of alcohol use but does not contribute the maintenance of binge-like ethanol drinking. It should be noted that the animals used to assess orexin-B IR drank lesser amounts of ethanol and sucrose and had lower resulting BECs relative to those animals used to assess orexin-A IR and OX

mRNA (Figure 2.2, 2.4, & 2.6). This difference in consumption likely stems from the different food diet that these animals were given during binge testing, which has been found to drastically affect ethanol consumption (Marshall *et al*, 2015). Despite the lower levels of consumption in these animals, we nonetheless observed group differences in orexin-B IR.

Although we observed a reduction in the number of OX peptide IR neurons following binge-like ethanol drinking, we did not observe a similar effect when we measured mRNA expression. Specifically, expression of the precursor, prepro-orexin, did not change following one or three cycles of binge-like ethanol consumption. However, mRNA expression significantly rose following three-, but not one-, cycles of sucrose DID. Similarly, we found that three-, but not one-, cycles of sucrose DID led to elevated OX1R mRNA expression within the amygdala. However, no significant change in OX1R mRNA was observed in the hypothalamus, VTA, or amygdala following binge-like ethanol drinking. Moreover, no alterations in OX2R mRNA were detected in the hypothalamus of any group. Together, these data indicate that production of OX peptides and receptors remain largely unaltered following binge-like ethanol consumption and suggest that binge-like ethanol drinking does not induce plastic adaptations in the OX system.

Previous studies have reported that ethanol consumption upregulates of the OX system by increasing prepro-orexin mRNA expression in the hypothalamus (Barson *et al*, 2015; Lawrence *et al*, 2006). Similarly, pharmacological studies indicate that ethanol consumption parallels OX signaling by showing that OX agonists increase ethanol consumption (Barson *et al*, 2015; Schneider *et al*, 2007) while antagonists reduce ethanol intake (Anderson *et al*, 2014; Jupp *et al*, 2011a; Lawrence *et al*, 2006; Moorman and Aston-Jones, 2009; Olney *et al*, 2015; Srinivasan *et al*, 2012). In light of these reports, we believe our observed reduction in OX peptide IR reflects

an increase in OX signaling. In this case, OX would be released from its presynaptic stores in response to consumption of salient reinforcers. As our IHC analysis measured the amount of peptide immediately following the last round of DID, a reduction in orexin-A and -B IR may be a sign of depleted stores of OX peptide due to an increase in signaling. Findings from our PCR analysis appear to support this hypothesis. Specifically, a reduction in prepro-orexin mRNA would have indicated a downregulation in the OX system following binge-like ethanol drinking. In this case, DID would have led to decreases in both OX peptide IR and precursor mRNA. However, we did not observe this to be the case. Instead, we found that expression of prepro-orexin mRNA remained unchanged following binge-like ethanol drinking. Thus, rather than a downregulated OX system, the reduced OX peptide IR is most likely a consequence of diminished OX levels due to increased release.

It should be noted that some details of the results from the PCR analysis were rather unexpected. First, we did not observe significant changes in hypothalamic prepro-orexin mRNA following binge-like ethanol consumption. This is inconsistent with the majority of the existing literature, which indicates that precursor mRNA is elevated following experience with ethanol. However, other reports exist that do not align with this pattern of results. For example, chronic ethanol consumption tends to increase OX mRNA (Barson *et al*, 2015; Lawrence *et al*, 2006). However, Morganstern and colleagues (2010) reported that acute ethanol caused an increase in OX expression while chronic ethanol consumption actually resulted in a significant reduction in hypothalamic OX mRNA. Given that different models of ethanol exposure can lead to differential effects on the OX system, it may be that binge-like ethanol drinking using the DID procedure simply does not lead to compensatory changes in the OX system. Alternatively, the timing of the tissue collection may have impacted the results as well. For example, we sacrificed our animals

immediately after the final binge session whereas others waited 30 min (Barson *et al*, 2015) or two hours (Morganstern *et al*, 2010) afterwards to assess OX mRNA. In the case of prepro-orexin expression we may have collected tissue before the system responded to the depleted levels of OX peptide and mobilized prepro-orexin mRNA to replenish those levels. Future studies may benefit from waiting longer after the final binge session to assess OX mRNA levels.

Furthermore, we did not expect expression of OX2R mRNA to be so low as to be largely undetectable in our PCR analysis. That being said, the pattern of regional OX2R expression- particularly the low expression of this receptor subtype- is consistent with the existing literature (Marcus *et al*, 2001). Other investigators have opted to pool tissue samples in order to augment the signal (Morganstern *et al*, 2010) and it remains to be seen whether a similar strategy would have resolved this issue in our analysis. In this case, combining tissue samples from mice with comparable drinking levels may have sufficiently improved the OX2R mRNA signal to allow for adequate statistical analysis and interpretation of subsequent results. However, we chose not to pool the samples in the current study as we only had 10 animals per group. Pooling the tissue may have improved the signal, but halving our sample size would have drastically reduced our statistical power- impeding our ability to detect statistical differences in OX expression.

Additionally, we observed a marked increase in both precursor and OX1R mRNA within the hypothalamus and amygdala, respectively, following 3 cycles of sucrose binge-like drinking. These data suggest that hypothalamic OX neurons that project to the amygdala are involved in sucrose consumption. Indeed, infusion of OX peptide directly into the amygdala greatly augments feeding behavior (Alò *et al*, 2015; Avolio *et al*, 2012; Rashmi *et al*, 2015). Importantly, this circuit has been specifically implicated in the consumption of highly palatable foods. In fact, schedule-induced feeding of a high-sugar diet causes a robust enhancement of

hypothalamic prepro-orexin mRNA expression relative to a standard diet (Olszewski *et al*, 2009). Together with the data from the current study, this suggests that a hypothalamic → amygdalar OX circuit is recruited following repeated access to highly palatable reinforcers, such as sucrose.

Findings from the present report indicate that binge-like ethanol consumption engages the hypothalamic OX system. The observed reduction in hypothalamic orexin-A and -B IR from our IHC experiments likely reflects increased signaling. This notion is supported by the results of our PCR analysis, which did not find any differences in OX mRNA due to binge-like ethanol consumption and suggests that the reduced levels of peptide were not a consequence of diminished production but rather a result of depleted peptide levels following release from the cell. Moreover, we found this effect not to be specific to ethanol as binge-like sucrose drinking resulted in a similar pattern of effects in terms of peptide IR. Interestingly, we found that repeated binge-like sucrose consumption caused a significant increase in hypothalamic prepro-orexin and amygdalar OX1R mRNA, which supports the idea that this circuit is recruited during the consumption of highly palatable food. Together, these data indicate that the OX system is perturbed following binge-like ethanol drinking in a DID model- specifically that binge drinking causes an increase in OX signaling. Experiments in the coming chapters will examine whether pharmacological blockade of this apparent increase in signaling in key brain regions innervated by OX neurons is capable of preventing binge-like ethanol drinking behavior.

**Figure 2.1**: The subregions of the hypothalamus investigated in the IHC experiments. 3V, third ventricle; D3V, dorsal third ventricle; DMH, dorsomedial hypothalamus; f, fornix; ic, internal capsule; LH, lateral hypothalamus; LV, lateral ventricle; mt, mammillothalamic tract; opt; optic tract; PFA, perifornical area of the hypothalamus; sox, supraoptic decussation.

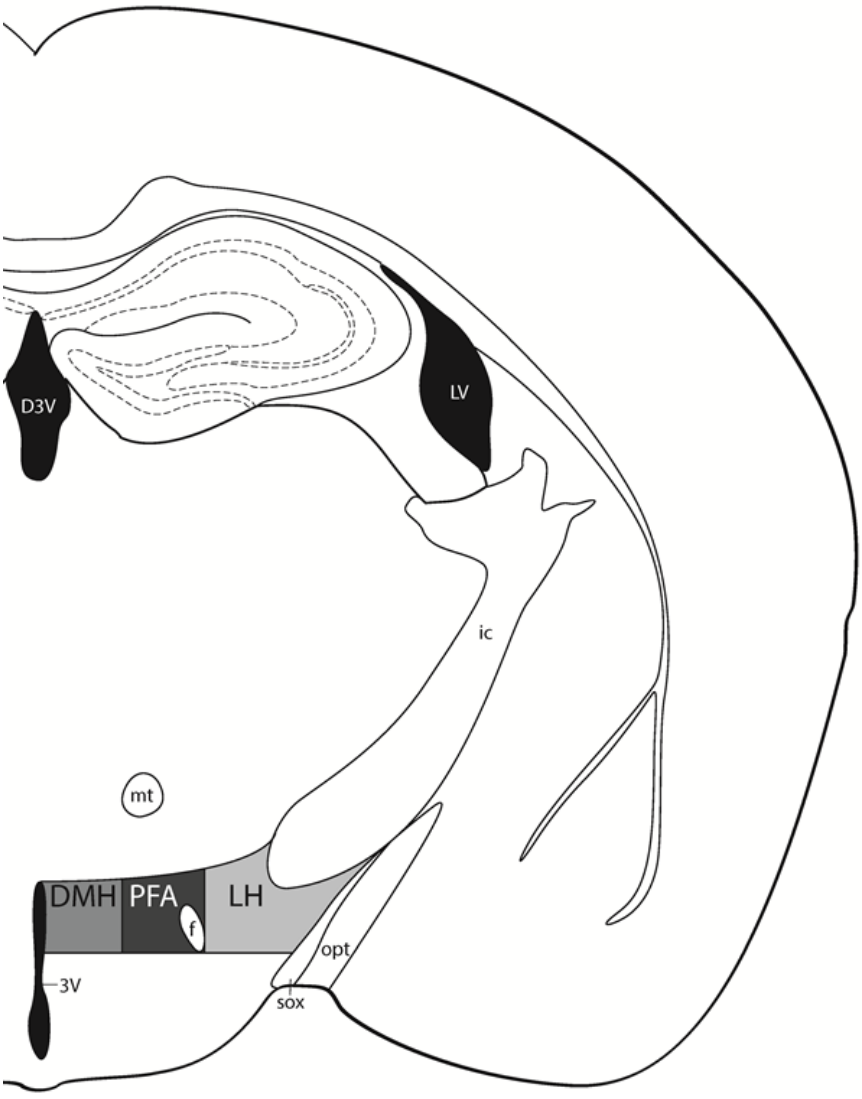


**Figure 2.2:** In the orexin-A IHC experiment, mice with access to ethanol bottles drank comparable levels of ethanol on the final day of testing regardless of whether they experienced one or three cycles of DID (A) and achieved similar BECs (B). Similarly, mice that experienced one or three cycles of DID consumed equivalent levels of sucrose (C). No differences were observed in final body mass as a function of group (D). H2O, water group; 1E, one-cycle ethanol group; 3E, three-cycle ethanol group; 1S, one-cycle sucrose group; 3S three-cycle sucrose group. Dashed line in (B) represents 80 mg/dl. Data are presented as Mean ± SEM.

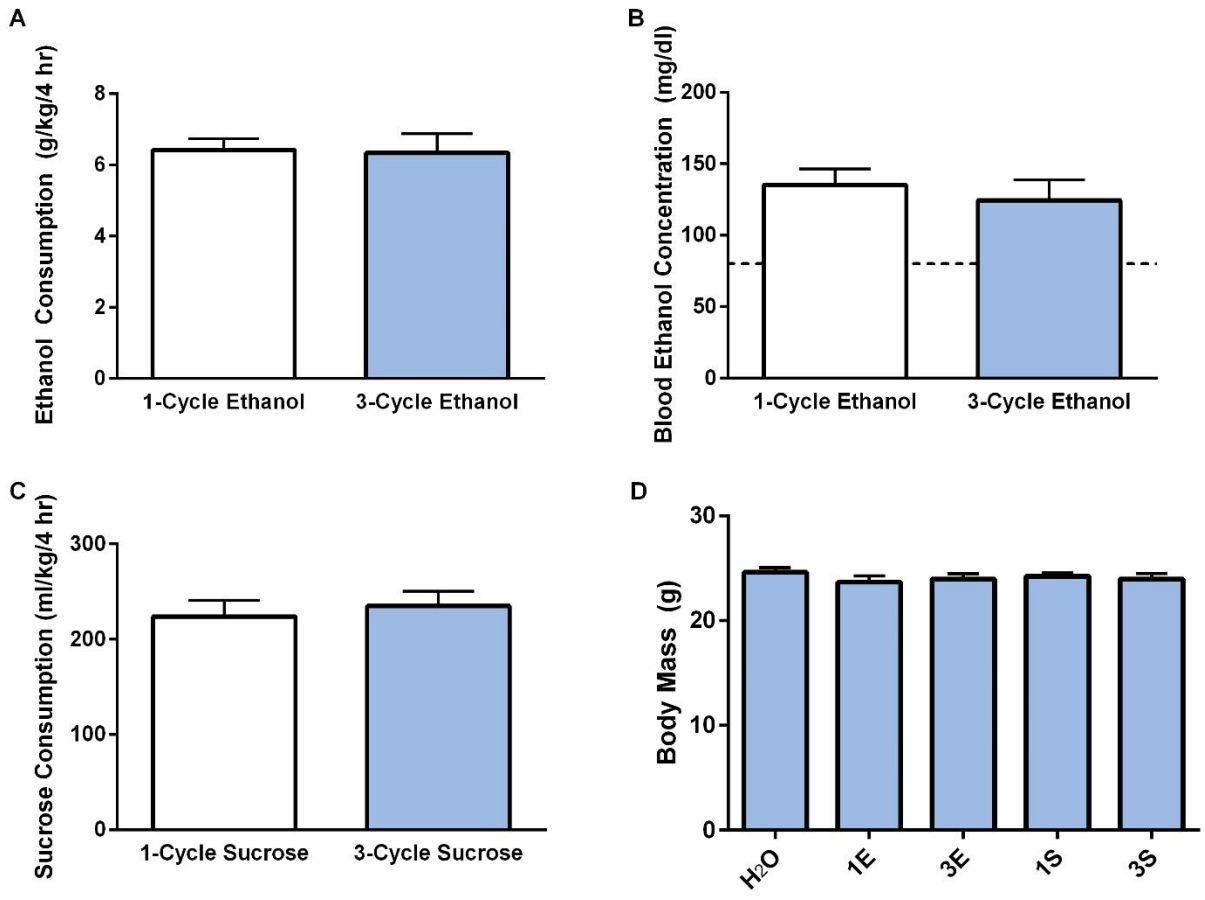
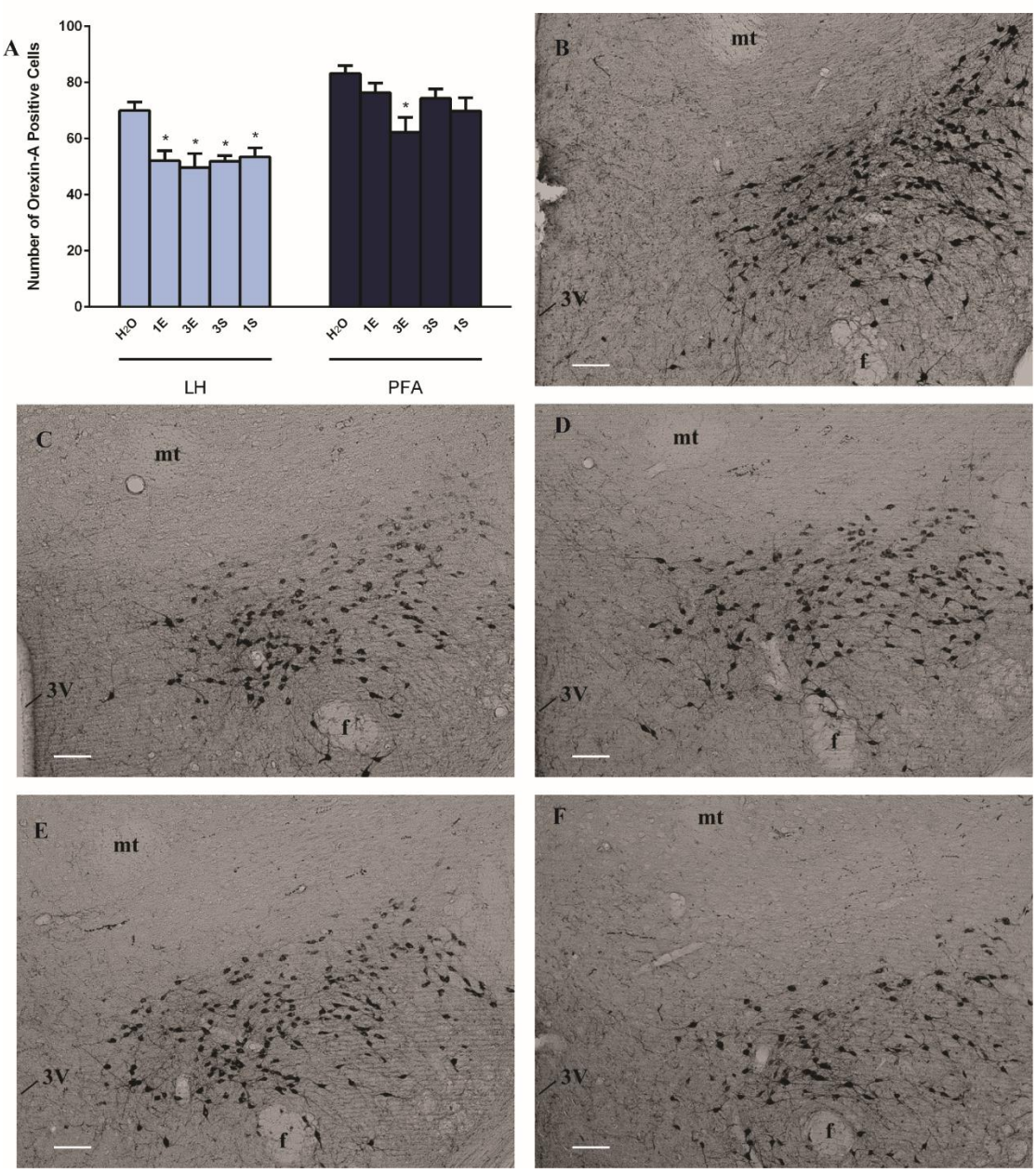


**Figure 2.3**: Assessment of hypothalamic orexin-A immunoreactivity (A) revealed that, relative to animals with access to water (B), binge-like ethanol drinking caused a reduction in orexin-A levels in the LH of animals that experienced one (C) or three cycles of ethanol (D) as well as one (E) or three cycles of sucrose (F). However, in the PFA, only animals that experienced three cycles of ethanol displayed significant reductions in levels of orexin-A while those that experienced one-cycle of ethanol as well as one or three cycles of sucrose exhibited no alterations in orexin-A IR in the PFA. LH, lateral hypothalamus; PFA, perifornical area of the hypothalamus; $H_2O$, water group; 1E, one-cycle ethanol group; 3E, three-cycle ethanol group; 1S, one-cycle sucrose group; 3S three-cycle sucrose group; * denotes $p < .05$ relative to H2O group in the same region. Horizontal white bar in B-F = 10.0 µm. Data are presented as Mean ± SEM.


A
Number of Orexin-A Positive Cells
100
80
60
40
20
0
H2O
1E
3E
3S
1S
LH
PFA
B
mt
3V
f
C
mt
3V
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D
mt
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f

**Figure 2.4:** In the orexin-B IHC experiment, mice with access to ethanol bottles drank comparable levels of ethanol on the final day of testing regardless of whether they experienced one or three cycles of DID (A) and achieved similar BECs (B). Similarly, mice that experienced one or three cycles of DID consumed equivalent levels of sucrose (C). No differences were observed in final body mass as a function of group (D). H2O, water group; 1E, one-cycle ethanol group; 3E, three-cycle ethanol group; 1S, one-cycle sucrose group; 3S three-cycle sucrose group. Dashed line in (B) represents 80 mg/dl. Data are presented as Mean ± SEM.

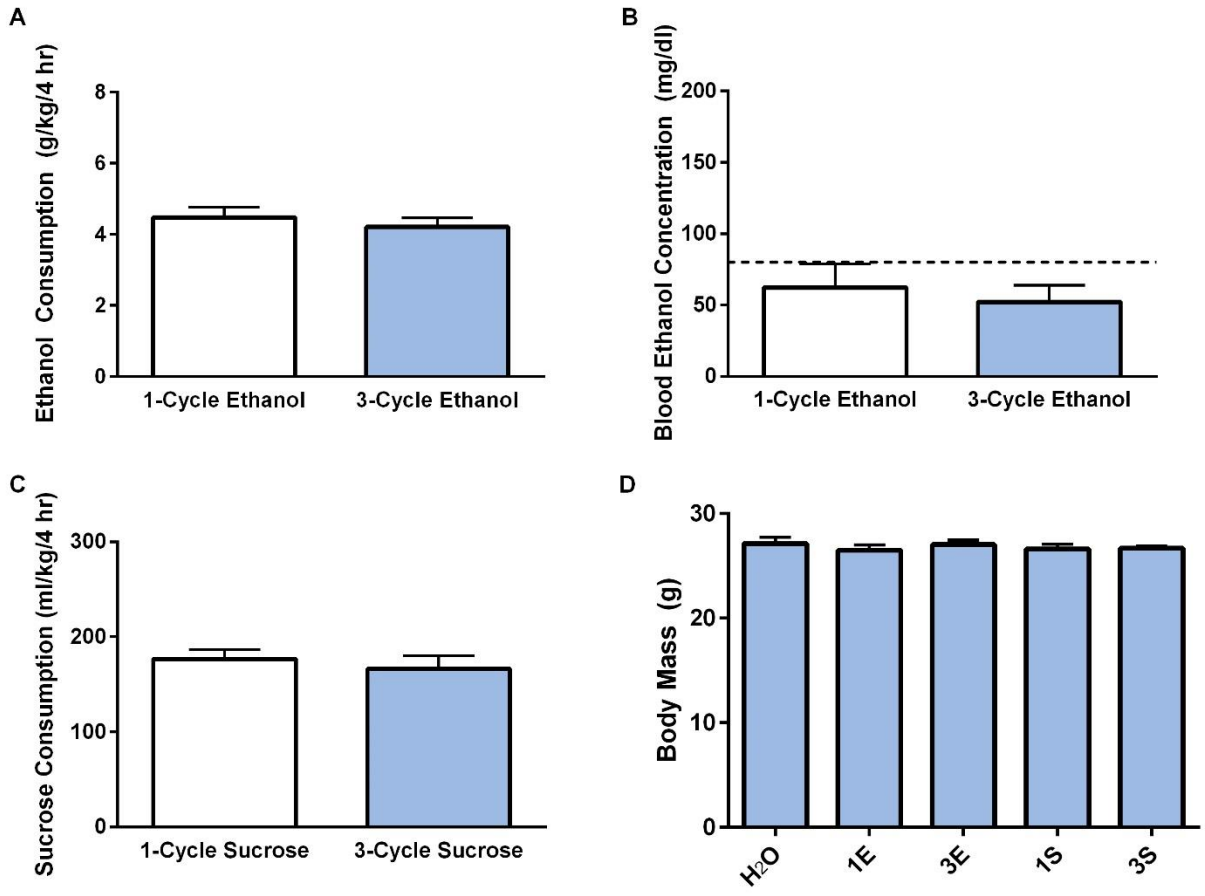


**Figure 2.5**: Assessment of hypothalamic orexin-B immunoreactivity (A) revealed that, relative to animals with access to water (B), binge-like ethanol drinking caused a reduction in orexin-B levels in the LH of animals that experienced one-cycle of ethanol (C), but levels returned to normal in animals that went through three-cycles of ethanol DID (D). In the PFA, a significant reduction in the number of orexin-B positive cells was observed in animals that experience one-cycle of either ethanol or sucrose (E); however, peptide levels returned to normal for each of the three-cycle ethanol or sucrose (F) groups. LH, lateral hypothalamus; PFA, perifornical area of the hypothalamus; $H_2O$, water group; 1E, one-cycle ethanol group; 3E, three-cycle ethanol group; 1S, one-cycle sucrose group; 3S three-cycle sucrose group; * denotes $p < .05$ relative to H2O group in the same region. Horizontal black bar in B-F = 10.0 µm. Data are presented as Mean ± SEM.

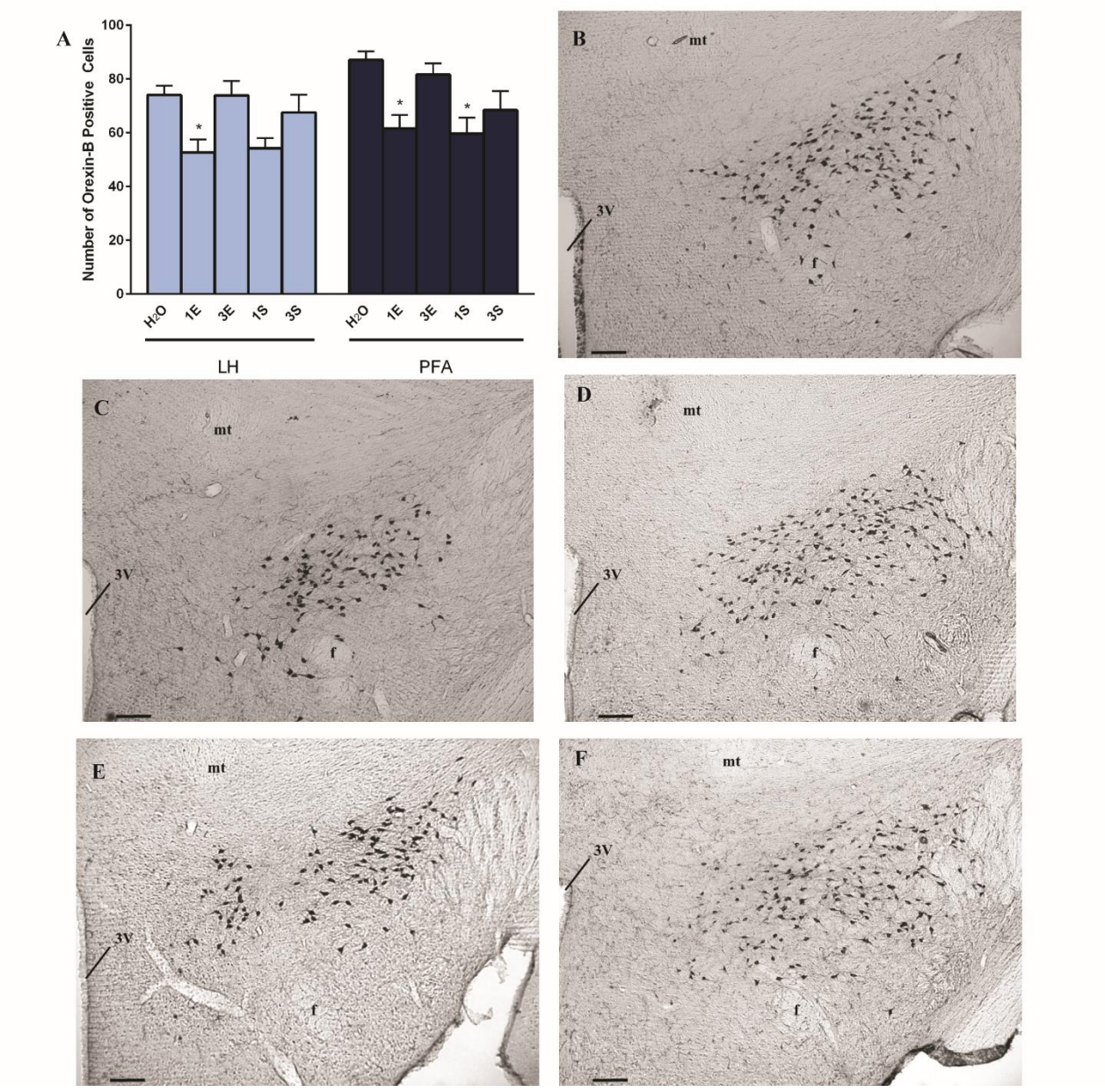

A
Number of Orexin-B Positive Cells
100
80
60
40
20
0
H2O
1E
3E
1S
3S
H2O
1E
3E
1S
3S
LH
PFA
B
mt
3V
f
C
mt
3V
f
D
mt
3V
f
E
mt
3V
f
F
mt
3V
f

**Figure 2.6:** In the PCR experiment, mice with access to ethanol bottles drank comparable levels of ethanol on the final day of testing regardless of whether they experienced one or three cycles of DID (A) and achieved similar BECs (B). Similarly, mice that experienced one or three cycles of DID consumed equivalent levels of sucrose (C). No differences were observed in final body mass as a function of group (D). H2O, water group; 1E, one-cycle ethanol group; 3E, three-cycle ethanol group; 1S, one-cycle sucrose group; 3S three-cycle sucrose group. Dashed line in (B) represents 80 mg/dl. Data are presented as Mean ± SEM.

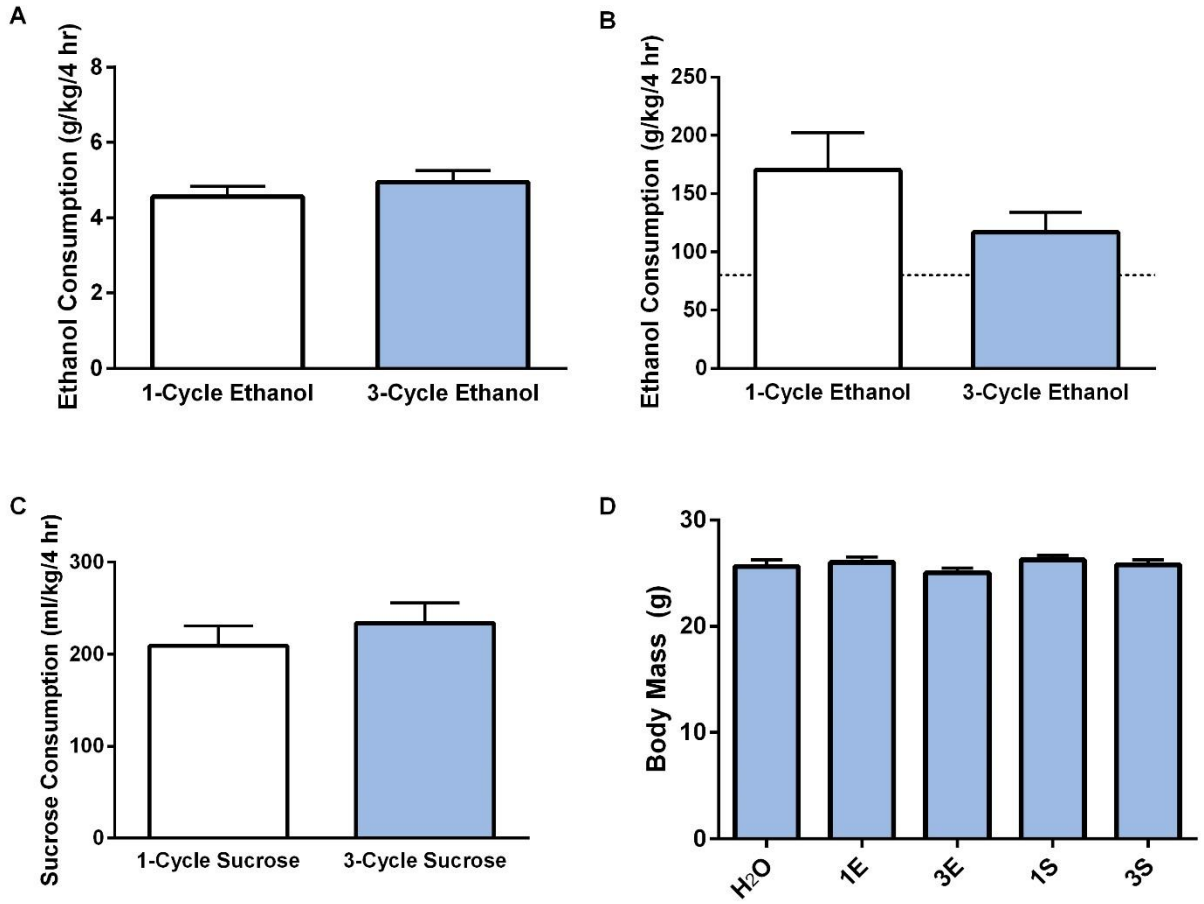
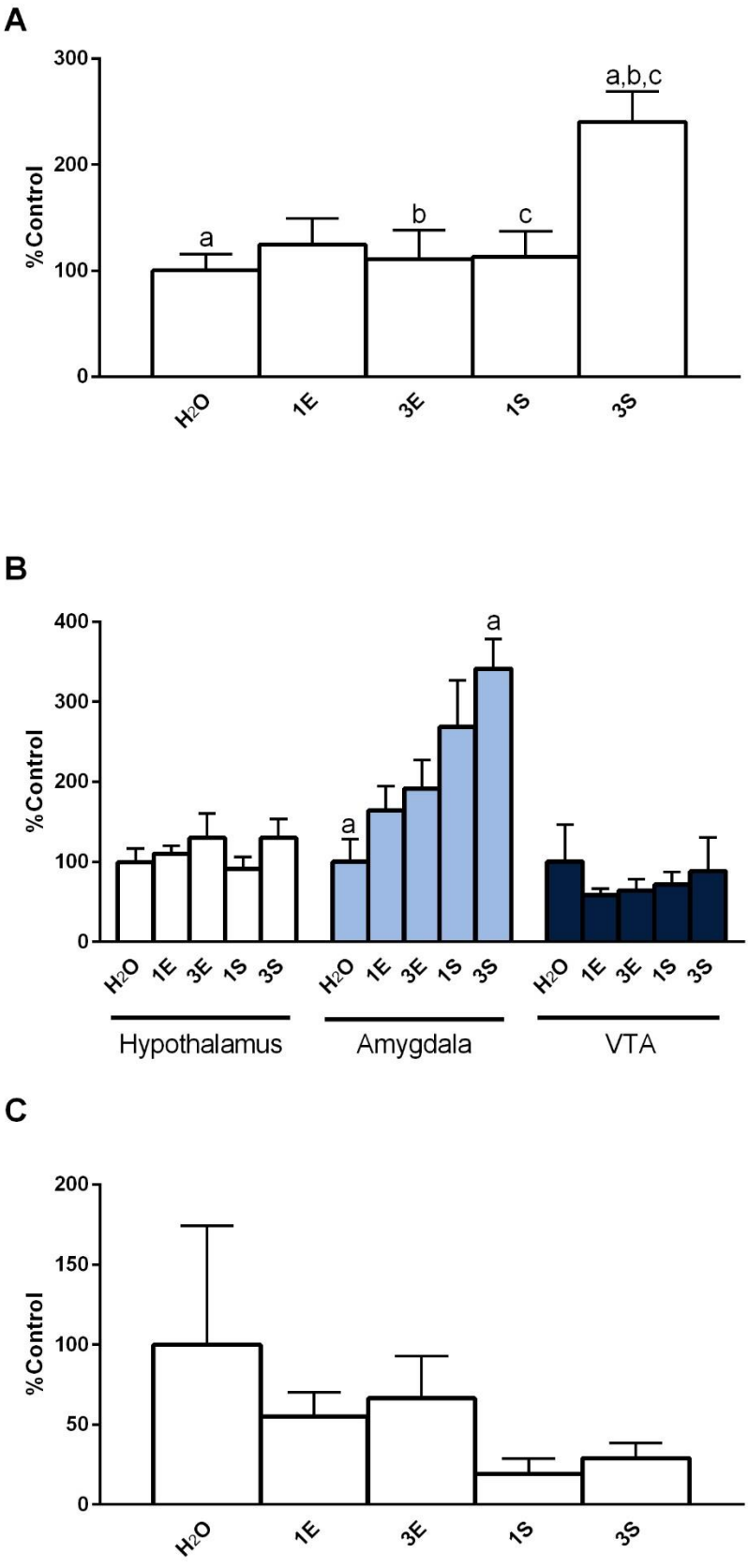


**Figure 2.7:** Expression of prepro-orexin within the hypothalamus (A); OX1R within the hypothalamus, amygdala, and VTA (B); and OX2R within the hypothalamus (C) following binge-like ethanol drinking. RT-PCR analysis revealed that the three cycles of binge-like sucrose consumption produced a marked increase in prepro-orexin mRNA expression within the hypothalamus relative to all other groups except for the one-cycle ethanol group (A). Similarly, the three-cycle ethanol group also showed a robust rise in OX1R mRNA expression within the amygdala relative to water drinking controls (B)- though no other changes in expression were observed in the hypothalamus or VTA. We were only able to detect the presence of OX2R mRNA in the hypothalamus, but no significant alterations in expression were observed as a function of group (C). H2O, water group; 1E, one-cycle ethanol group; 3E, three-cycle ethanol group; 1S, one-cycle sucrose group; 3S three-cycle sucrose group. Bars that share the same letter are significantly different from one another ($p < 0.05$). Data are presented as Mean ± SEM.


A
300
200
100
0
%Control
a
b
c
a,b,c
H2O
1E
3E
1S
3S
B
400
300
200
100
0
%Control
a
a
H2O
1E
3E
1S
3S
Hypothalamus
Amygdala
VTA
C
200
150
100
50
0
%Control
H2O
1E
3E
1S
3S

# CHAPTER 3

# PHARMACOLOGICAL INVESTIGATION OF THE ROLE OF OREXIN SIGNALING WITHIN REWARD-RELATED CIRCUITRY IN BINGE-LIKE ETHANOL DRINKING BEHAVIOR

## Introduction

The OX system is comprised of two peptides, orexin-A and -B, that act on two GPCRs, OX1R and OX2R. Orexin-A has been found to bind with a relatively high affinity for both receptors while orexin-B mainly interacts with the OX2R as it has a relatively low affinity for the OX1R (Sakurai *et al*, 1998). Although neurons that produce the OX peptides are found exclusively within the hypothalamus, they project to various regions throughout the brain to regulate a host of neurobiological functions (Ch'ng and Lawrence, 2015; Peyron *et al*, 1998; Yoshida *et al*, 2005). Indeed, relatively recent evidence has emerged that has implicated the OX system in modulating the responses to drugs of abuse, including ethanol (see Mahler *et al*, 2012 for review).

It was initially believed that only the OX1R would modulate ethanol responding (Harris and Aston-Jones, 2006) based on a limited amount of data at the time; however, later research revealed that the OX2R is also capable of modulating ethanol drinking (Anderson *et al*, 2014; Barson *et al*, 2015; Shoblock *et al*, 2011). In fact, a clear pattern emerges across the literature that indicates that ethanol drinking parallels OX signaling such that OXR antagonists blunt

ethanol consumption while OXR agonists enhance drinking behavior (Jupp *et al*, 2011a; Lawrence *et al*, 2006; Moorman and Aston-Jones, 2009; Schneider *et al*, 2007).

Despite this growing body of literature implicating the OX system in ethanol consumption, relatively less research has been dedicated to elucidating its role in binge-like ethanol drinking behavior. Indeed, we and others have recently reported that peripheral administration of either an OX1R or OX2R antagonist reduces binge-like ethanol drinking using the "drinking in the dark" (DID) model (Anderson *et al*, 2014; Olney *et al*, 2015). Notably, these investigations also found that OXR antagonists affect palatable reinforcers regardless of caloric content by demonstrating that these compounds also reduced binge-like sucrose (Anderson *et al*, 2014) and saccharin consumption (Alcaraz-Iborra *et al*, 2014; Olney *et al*, 2015). These observations indicate that peripherally administered OXR antagonists do not selectively modulate ethanol consumption *per se*, but rather regulates the underlying responses to general, salient reinforcers, which includes ethanol.

These studies, however, were unable to identify the brain regions that govern this effect due to the systemic nature of the treatment procedure. Considering its critical role in reward processing, a potential region that may play a substantial role in this effect is the VTA. Indeed, engaging in reinforcing behavior has been shown to increase activity in the VTA in animals (Esposito *et al*, 1984; Porrino *et al*, 1984) and humans (Breiter *et al*, 1997) while inactivation of the VTA results in blunted responding to a sucrose reward (van Zessen *et al*, 2012). Together, these studies and many others evince the VTA as a likely participant in reinforcing behavior, such as binge ethanol drinking.

What is more, the VTA is densely innervated by hypothalamic OX neurons (Ch'ng and Lawrence, 2015; Peyron *et al*, 1998; Yoshida *et al*, 2005) and expresses both OXR subtypes (Marcus *et al*, 2001; Narita, 2006). OX peptides acting on either OXR subtype within the VTA results in a robust depolarization of VTA neurons (Korotkova *et al*, 2003) and is accompanied by a marked increase in DA release as well (España *et al*, 2010; Narita, 2006; Vittoz *et al*, 2008). Moreover, both orexin-A and -B have even been shown to cause plastic changes in dopaminergic neurons within the VTA that facilitates activity in this region (Borgland *et al*, 2006, 2008). Considering these findings, it is not surprising that infusions of a dual OXR antagonist directly into the VTA has been demonstrated to disrupt operant responding to ethanol in rats (Srinivasan *et al*, 2012). As a whole, these observations implicate OX neurons- originating from the hypothalamus- that project to the VTA as the prime circuit that modulates binge-like ethanol drinking.

The purpose of the present study was to more definitively elucidate the contribution of OX signaling within the VTA in binge-like drinking behavior. Because previous investigations into the role of OX signaling within the VTA in ethanol responding used a nonselective OXR antagonist (Srinivasan *et al*, 2012), we also sought to determine the individual OXR subtypes involved in binge drinking by utilizing selective compounds that act on either the OX1R or OX2R. Here, we show that inhibition of the OX1R, but not OX2R, in the VTA selectively reduces binge-like ethanol drinking without altering anxiety-like behavior in the animals. These data suggest that OX signaling onto OX1Rs within the VTA modulates binge-like ethanol drinking.

## Methods

### *Animals*

Male C57BL/6J mice (C57; Jackson Laboratories, Bar Harbor, ME), aged 6-7 weeks and weighing 20-25 g upon arrival, were used in each of the following studies. Mice were individually housed in plastic cages located in a vivarium with an ambient temperature of approximately 22ºC and a reverse light/dark cycle with lights off at 8:30 am. All animals had *ad libitum* access to food and water except when specified below. All procedures used were in accordance with the National Institute of Health guidelines and were approved by the University of North Carolina Institutional Animal Care and Use Committee.

### *Cannulation Surgery and Infusion Procedures*

Prior to testing, mice were bilaterally cannulated targeting the VTA (AP: -3.08 mm, ML: ±0.50 mm, DV: -4.40 mm). As a regional control, a separate group of mice was cannulated slightly dorsal to the VTA (AP: -3.08 mm, ML: ±0.50 mm, DV: -3.40 mm). Mice were given one week to recover from surgery before testing. Cannula placement for each animal was verified histologically and animals in which the cannulas were not in the target area were excluded from the statistical analysis (see Figure 3.1).

On test days, mice were infused with either the selective OX1R antagonist, SB-334867 (SB; 0.0 or 6.0 µg; Tocris Bioscience, Bristol, UK), or the selective OX2R antagonist, TCS-OX2-29 (TCS; 0.0, 5.0, or 7.5 µg; Tocris Bioscience, Bristol, UK). These doses were determined based on what has been demonstrated to be effective previously (Borgland *et al*, 2006; Li *et al*, 2011). SB is 50-fold selective for the OX1R over the OX2R (Porter *et al*, 2001; Smart *et al*, 2001) while TCS has a 250-fold selectivity for the OX2R over the OX1R (Hirose *et*

*al*, 2003). Both compounds were dissolved in dimethyl sulfoxide (DMSO) to reach the desired concentration. DMSO was chosen as the solvent for both drugs in order to better equate the actions of SB and TCS. Importantly, a dose higher than 7.5 µg of TCS could not be achieved due to restrictions in the drug's solubility in DMSO. All infusions were administered in a volume of 0.3 µl per side and were delivered over the course of one minute using an automated syringe pump (Harvard Apparatus, Holliston, MA). Injectors were left in the cannulae for an extra minute before removal to ensure adequate diffusion of the compound and to prevent reflux up the cannula tract. Drug infusions for all studies were performed 30 min prior to the start of the behavioral test.

*Drinking in the Dark*

A cohort of 17 C57BL/6J (C57) mice was used to assess the effect of pharmacological inhibition of VTA OX1Rs on binge-like ethanol and sucrose consumption. A separate group of 22 mice was used to similarly assess inhibition of VTA OX2Rs. Additionally, another cohort of 12 mice was used to verify the specificity of the effect by infusing the OX1R antagonist into a control region 1.0 mm dorsal to the VTA. The DID procedure is a commonly used animal model of binge-like ethanol drinking that promotes high levels of consumption and generates physiologically relevant blood ethanol concentrations (BECs) of 80 mg/dl or greater (Rhodes *et al*, 2005, 2007). On days 1-3, standard water bottles were removed three hours into the dark cycle and the animals were given access to a single test bottle containing ethanol (20% v/v) or sucrose (3% w/v) solutions for two hours. Binge-like intake was assessed on the fourth day in which test bottles were measured hourly in order to examine the effect of the drug on consumption over time. Immediately after test bottles were removed on the fourth day of ethanol testing, tail-blood samples were taken from each animal and processed to determine blood

ethanol concentration (BEC) using an alcohol analyzer (Analox Instruments, London, UK). A two hour test period was chosen as we have previously had success capturing the transient effects of the drug using this shortened procedure (Olney *et al*, 2015). In order to increase power during statistical analysis, a Latin-square design was used such that each animal received all doses of the drug over repeated trials. Mice were given three days of rest between subsequent 4-day DID sessions in order to avoid carryover effects of the drug.

*Elevated Zero Maze*

A separate cohort of 12 C57s was used to assess the effect of pharmacological inhibition of VTA OX1Rs on anxiety-like behavior using a five minute test on the elevated zero maze (Med Associates, Inc., St. Albans, VT). The elevated zero maze is a common tool used among investigators in order to assess anxiety-like behavior. Here, anxiolytic drugs increase the amount of time spent in the open area while anxiogenic drugs increase the amount of time spend in the closed areas (Shepherd *et al*, 1994). One hour before testing, animals were brought into a dark room that housed the elevated zero maze. Approximately three hours into the dark cycle, testing on the elevated zero maze began by placing the animal in the open area of the maze. Each session was recorded using a camcorder placed above the apparatus and was scored by an investigator blind to the animal's group assignment who recorded the time (s) spent in the open area as well as the number of entries into the open and closed area. The animal was considered to have entered the open area when all four paws left the closed area. Open area time was considered terminated once all of the animal's paws entered the closed area. We also assessed the number of instances and time (s) spent "exploring" the open area from the closed area. Exploring behavior was defined as each time the animal, while still in the closed area, extended its head beyond the ears into the open area. Exploring behavior ceased when the animal either

entered the open area or retreated back into the closed area. Animals were returned to their homecage immediately after the conclusion of the test session. Unlike DID testing, a Latin-square design was not used in order to avoid previous experience with the apparatus confounding the animals' behavior. Treatment groups were equated based on body weight and each animal received a single, bilateral infusion of either 0.0 or 6.0 μg of SB.

*Open-Field Locomotor Activity*

The same group of animals from the previous elevated zero maze test was used to assess the effect of pharmacological inhibition of VTA OX1Rs on locomotor activity; however, one animal's cannula became clogged in the interim time between tests and was unable to be infused. Thus, only 11 C57s were used. In addition to assessing general locomotor activity in the animals, this test is capable of measuring anxiety-like behavior as well. Similar to the elevated zero maze, treatment with anxiolytic compounds generally causes the animal to increase the amount of time spent in the center of the open-field chamber (Choleris *et al*, 2001). Testing in the open-field locomotor chambers occurred 48 h after testing in the elevated zero maze. One hour before testing, animals were brought into a dark room adjacent to the room that housed the locomotor chambers. Three hours into the dark cycle, animals were placed in a 16.5 x 16.5 $in^2$ open-field locomotor chamber (Accuscan Instruments, Columbus, OH) and locomotor activity was recorded in five minute bins for one hour using VersaMax software (Omnitech Electronics, Columbus, OH). This software was able to record total distance traveled (cm) as well as the time (s) spent and distance traveled (cm) in the center or margin of the chamber. Using these measures, we calculated separate variables for the percent time spent in the center of the chamber ([time spent in the center ÷ (time spent in the center + time spent in the margins)] × 100) as well as the percent distance traveled in the center of the chamber ([distance traveled in the center ÷

(distance traveled in the center + distance traveled in along the margins)] × 100). Mice were placed back into their homecages after the one hour test period. Like the elevated plus maze, a Latin-square design was not used for the locomotor test. Animals received a single, bilateral infusion of either 0.0 or 6.0 µg of SB. Each animal received the alternate drug treatment based on its assigned drug condition during the previous elevated zero maze test.

*Data Analysis*

A repeated-measures ANOVA was used to assess hourly binge consumption with both time (hour 1 and hour 2) and dose (0.0 or 6.0 µg for SB; 0.0, 5.0, or 7.5 µg for TCS) being within-subject variables. Additionally, BEC and total binge consumption across the two-hour test period was assessed using separate repeated-measures ANOVAs with dose (0.0 or 6.0 µg for SB; 0.0, 5.0, or 7.5 µg for TCS) as the within-subject variable. We also included drug order as a between-subjects variable in these analyses to ensure the order in which the animals were presented the drug did not have any confounding effects on drinking behavior. Importantly, due to the relatively short half-life and hyper-transient nature of the compounds (Mould *et al*, 2014; Porter *et al*, 2001), the effects of these compounds can be rather short-lived (Olney *et al*, 2015). Thus, planned comparisons were used to assess binge-like consumption of each drug group relative to its respective vehicle during the first hour of testing in order to better capture the short-lived effect of the compound.

Additionally, separate univariate ANOVAs used dose of SB (0.0 or 6.0 µg) to predict the time spent in the open area, the number of entries into the open area, time spent exploring the open area, and frequency of exploratory behavior in the elevated plus maze paradigm. Similarly, separate repeated-measures ANOVAs were used to assess behavior in the open-field locomotor

tests with dose of SB (0.0 or 6.0 μg) being a between-subjects variable while total distance traveled, percent distance traveled in the center, and percent time spent in the center across the twelve 5-min bins were all considered within-subject variables. Tukey's LSD *post-hoc* tests and Bonferroni corrections were employed throughout the analyses when applicable.

**Results**

Our omnibus analysis revealed that binge-like ethanol consumption levels did not change over time (Figure 3.2A; Main effect of Time: $F_{(1,15)} = 2.701$, $p = 0.121$) nor did inhibition of OX1Rs in the VTA significantly alter binge-like ethanol drinking (Main effect of Dose: $F_{(1,15)} = 2.651$, $p = 0.096$; Time×Dose Interaction: $F_{(1,15)} = 2.189$, $p = 0.160$). However, planned comparisons revealed that, relative to vehicle treated controls, SB significantly blunted binge-like ethanol intake during the first hour of testing ($t_{(16)} = 2.208$, $p = 0.042$). Moreover, no significant effect was observed in total binge-like ethanol drinking across the two-hour test period ($F_{(1,15)} = 3.161$, $p = 0.096$). Despite the reduction in binge drinking during the first hour of testing, no significant difference in BECs was observed between the two drug conditions when blood samples were collected after the full two hour test (Figure 3.2B; $F_{(1,15)} = 0.419$, $p = 0.527$). Importantly, binge-like ethanol drinking was not affected by the order in which the animals received their treatment ($F_{(1,15)} = 3.489$, $p = 0.081$) nor did it impact BECs ($F_{(1,15)} = 0.134$, $p = 0.720$).

Looking next at binge-like sucrose consumption, there was a trend for animals to consume more sucrose during the first hour of testing relative to the second but the effect did not reach significance (Figure 3.2C; Main effect of Time: $F_{(1,15)} = 4.253$, $p = 0.064$). Binge-like sucrose drinking was not impacted as a function of SB treatment (Main effect of Dose: $F_{(1,15)} =$

0.711, $p = 0.412$); however, we did find a significant interaction effect (Time×Dose Interaction: $F_{(1,15)} = 4.876$, $p = 0.043$). Further probing revealed that this effect was driven by the fact that binge-like sucrose intake among vehicle-treated animals was significantly greater during the first hour of testing relative to the second hour ($t_{(16)} = 2.473$, $p = 0.025$). Notably, planned comparisons revealed no significant difference in sucrose consumption during the first hour of testing between the two treatment groups ($t_{(16)} = 1.463$, $p = 0.163$). Moreover, sucrose consumption across the entire two hour test period did not significantly vary as a function of SB treatment ($F_{(1, 15)} = 0.711$, $p = 0.412$). Additionally, the order in which the animal received the compound did not confound binge-like sucrose drinking ($F_{(1, 15)} = 0.024$, $p = 0.878$).

Importantly, we found the effect of intra-VTA SB to blunt binge-like ethanol consumption to be specific to the VTA as similar treatment slightly dorsal to the VTA did not produce any significant alterations in binge-like ethanol drinking as a function of treatment (Figure 3.2D; Main effect of Dose: $F_{(1,10)} = 4.188$, $p = 0.068$; Time×Dose Interaction: $F_{(1,10)} = 3.989$, $p = 0.074$; Total ethanol consumption: $F_{(1,10)} = 4.188$, $p = 0.068$; SB versus vehicle planned comparison at the first hour: $t_{(11)} = -0.278$, $p = 0.786$) although we did observe that the animals drank significantly more ethanol during the second hour of testing relative to the first hour regardless of treatment condition (Main effect of Time: $F_{(1,10)} = 7.037$, $p = 0.024$). Moreover, we did not observe any effect on BEC levels as a function of treatment (Figure 3.2E; $F_{(1,9)} = 0.028$, $p = 0.870$). Additionally, whether the animal received treatment with vehicle or SB first did not impact binge-like ethanol consumption ($F_{(1,10)} = 0.268$, $p = 0.616$) or BECs ($F_{(1,9)} = 0.108$, $p = 0.750$).

We next sought do determine the role of intra-VTA OX2Rs in binge-like ethanol drinking. Repeated-measures ANOVA revealed that there was a marginal effect of ethanol

drinking over time but the effect did not reach significance (Figure 3.3A; Main effect of Time: $F_{(1,19)} = 3.175$, $p = 0.091$). Similarly, none of the doses tested affected binge-like ethanol intake (Main effect of Dose: $F_{(2,38)} = 0.669$, $p = 0.518$; Time×Dose Interaction: $F_{(2,38)} = 0.425$, $p = 0.657$). Using planned comparisons, we did not observe any significant effect in the low dose (5.0 µg) of TCS relative to vehicle-treated controls during the first hour of testing ($t_{(21)} = -0.005$, $p = 0.996$) but we did see a marginal, yet nonsignificant, trend for the high dose (7.5 µg) of TCS to reduce binge-like ethanol consumption relative to vehicle ($t_{(21)} = 1.790$, $p = 0.088$). Additionally, we did not observe any effect across the total two hours of testing ($F_{(2,38)} = 0.669$, $p = 0.518$). Although we did not observe any significant alterations in drinking behavior, there was a marginal, albeit nonsignificant, effect for BEC levels to vary as a function of treatment group (Figure 3.3B; $F_{(2,34)} = 3.181$, $p = 0.054$). Moreover, the order in which the animals received the drug treatment did not significantly impact binge-like ethanol drinking ($F_{(1,\ 19)} = 0.194$, $p = 0.825$).

It is well-known that both positive and negative reinforcement may drive an individual to consume ethanol (Eckardt *et al*, 1998; Koob, 2009; Sinha, 2007). The VTA is perhaps best known as a brain area that is integral to reward processing; however, recent evidence has emerged that indicates that OX activity in the VTA may drive stress responding as well (Hata *et al*, 2011). In an effort to disentangle the overlaying psychological drive that explains how inhibition of OX1Rs in the VTA ultimately leads to blunted binge-like ethanol drinking we next sought to explore how intra-VTA treatment with SB affects anxiety-like behavior. Using the elevated zero maze, we found that silencing signaling onto OX1Rs within the VTA did not alter the time spent in the open area (Figure 3.4A; $F_{(1,\ 10)} = 1.169$, $p = 0.305$) or the number entries into the open area (Figure 3.4B; $F_{(1,\ 10)} = 0.002$, $p = 0.964$). What is more, the drug impacted

neither the frequency that the animal explored the open area (Figure 3.4D; $F_{(1, 10)} = 1.901$, $p = 0.198$) nor the time engaged in exploratory behavior ($F_{(1, 10)} = 1.443$, $p = 0.257$).

Similarly, we did not observe any effect of intra-VTA SB on anxiety-like behaviors as measured in the open-field locomotor test. Inhibiting OX1Rs within the VTA did not alter the proportion of distance traveled in the center of the chamber relative to the margins (Figure 3.5A; Main effect of Dose: $F_{(1, 8)} = 0.740$, $p = 0.415$; Time×Dose Interaction: $F_{(11,88)} = 1.425$, $p = 0.254$); although the animals did display significant variability in the proportion of distance traveled in the center as a function of time (Main effect of Time: $F_{(11,88)} = 4.573$, $p = 0.007$). Further probing of this effect with Tukey's LSD revealed the significant differences at the following time points: 10 min > 40 min ($p = 0.002$), 10 min > 55 min ($p = 0.001$), 15 min > 25 min ($p = 0.002$), 15 min > 55 min ($p = 0.003$), 20 min > 55 min ($p = 0.003$), 35 min > 45 min ($p = 0.002$), 35 min > 50 min, ($p = 0.004$), 35 min > 55 min ($p = 0.001$). We also did not observe any significant effects in any of the variables assessing the proportion of time spent in the center of the chamber relative to the margins (Figure 3.5B; Main effect of Time: $F_{(11,88)} = 1.223$, $p = 0.318$; Main effect of Dose: $F_{(1, 8)} = 0.156$, $p = 0.704$; Time×Dose Interaction: $F_{(11,88)} = 0.700$, $p = 0.617$). Notably, intra-VTA treatment with SB did not significantly influence gross locomotor activity (Figure 3.5C; Main effect of Dose: $F_{(1, 8)} = 2.122$, $p = 0.183$; Time×Dose Interaction: $F_{(11,88)} = 1.472$, $p = 0.231$); although the animals exhibited varying amounts of activity across the 60 min test period (Main effect of Time: $F_{(11,88)} = 10.544$, $p < 0.0001$). *Post hoc* analyses of this effect revealed that the animals were most active towards the beginning of the test (5 min > all other time points, $p$'s < 0.002; 10 min > 20 min, $p = 0.001$; 10 min > 60 min, $p = 0.004$).

**Discussion**

It has previously been reported that peripheral administration of either an OX1R or OX2R antagonist is capable of protecting against binge-like consumption of a salient reinforcer (Anderson *et al*, 2014; Olney *et al*, 2015), and that signaling onto the OX1R or OX2R (or both) within the VTA modulates ethanol responding (Srinivasan *et al*, 2012). Findings from the current report were able to provide a more detailed characterization of the contribution of OX signaling within the VTA in modulating binge-like ethanol drinking behavior. Specifically, we demonstrated that intra-VTA infusion of SB, but not TCS, reduced binge-like ethanol intake. Despite this effect, SB did not significantly reduce BECs; however, this may be due to the very short half-life (Porter *et al*, 2001) and the fact that BECs were measured at the end of the two hour test period. Moreover, this effect was found to be reinforcer- and brain region-specific as similar treatment with SB did not alter binge-like sucrose consumption nor did it impact binge-like ethanol drinking when infused dorsal to the VTA. Notably, inhibiting OX1Rs in the VTA did not impact general locomotor behavior (Figure 3.5C)-indicating that such treatment does not engage OX circuitry involved in sleep and arousal (de Lecea, 2012). Together, these experiments were able to extend the previous literature by observing OX signaling- specifically onto the OX1R- within the VTA, in part, modulates binge-like ethanol consumption without altering anxiety-like behaviors.

Notably, inhibiting intra-VTA OX2Rs via TCS failed to significantly alter binge-like ethanol drinking in our experiments across a range of doses. Although one may surmise that-based on our findings- OX2Rs within the VTA do not contribute to binge drinking behavior, it may be more appropriate to conclude that OX2Rs play a subsidiary role to OX1Rs in modulating ethanol drinking. Indeed, previous studies have demonstrated that the OX2R is capable of

modulating ethanol drinking (Anderson *et al*, 2014; Barson *et al*, 2015; Shoblock *et al*, 2011). More importantly, we did observe a slight, albeit nonsignificant, trend for the higher dose of TCS (7.5 µg) to disrupt ethanol drinking during the first hour of testing relative to vehicle-treated animals. Furthermore, we also observed that the lower dose of TCS (5.0 µg) reduced BECs to a level below the threshold to be considered a binge episode and marginally reduced BECs relative to vehicle-treated controls. Together, these findings suggest that OX2Rs in the VTA may have relatively more subtle effects on ethanol consumption that were not fully captured in the present experiments. Thus, it may be the case that signaling onto OX2Rs within the VTA contributes to binge-like ethanol drinking behavior but that its role is secondary to that of OX1Rs- although further testing of this hypothesis is needed before any definitive conclusions can be drawn. Although we used a range of doses in order to better assess the contribution of the OX2R, we were limited in the concentration of TCS we could feasibly use due to restrictions in the solubility of the compound in DMSO; thus, alternative OXR agents may be ideal for future investigations. For example, if the OX2R does contribute to this behavior then intra-VTA administration of orexin-B, which has a much greater affinity for the OX2R over the OX1R, may augment ethanol drinking behavior.

Perhaps the most intriguing observation was the fact that the reduction in binge-like drinking produced by intra-VTA treatment with SB was selective for ethanol intake. Contrary to the present results, numerous studies have reported that the effects of OXR antagonists are not specific to ethanol but rather more broadly affect responding to general, salient reinforcers (Alcaraz-Iborra *et al*, 2014; Anderson *et al*, 2014; Olney *et al*, 2015). This is not the first instance in which differential effects of OXR antagonists on responding to reinforcers has been observed as OXR antagonists have been shown to reduce operant responding to ethanol, but not

sucrose (Jupp *et al*, 2011a; Srinivasan *et al*, 2012). The ability of drugs of abuse to produce a rewarding experience in the organism is commonly conceptualized as being due to its ability to "hijack" the brain's existing natural reward system. In this sense, there is a great deal of overlap regarding the circuitry regulating natural rewards and that which modulates drugs of abuse. However, our findings and others suggest that a distinct OX circuit- likely involving the VTA- is recruited for ethanol consumption versus that of a natural reward. As a whole, these discoveries reveal that these pathways may be more segregated than originally believed- a notion that may hold great value when developing novel treatments for drug abuse.

Although it has been long-known that ethanol activates these reward circuits (Ingvar *et al*, 1998; Weiss *et al*, 1993), ethanol also engages stress/anxiety circuits to produce anxiolysis (Barkley-Levenson and Crabbe, 2015; Pandey *et al*, 2008; Wilson *et al*, 2004). Considering that both the positive- and negative-reinforcing effects of ethanol's neurobiological actions may serve to perpetuate drinking (Eckardt *et al*, 1998) and intra-VTA OX signaling modulates both reward processing (Taslimi *et al*, 2012) and stress responding (Hata *et al*, 2011), it was necessary to elucidate whether the effects observed in the present report were associated with alterations in stress responses, such as anxiety-like behavior. Results garnered from the current experiments revealed that intra-VTA SB does not impact anxiety-like behaviors as measured in either the elevated zero maze or open-field locomotor tests, suggesting that suppression of OX1R signaling, at least within the VTA, does not alter stress responding. Accordingly, these data suggest that OX1R inhibition in the VTA blunts binge-like ethanol drinking independent of changes in anxiety-like behaviors.

In summary, findings from the present report suggest that OX1R signaling within the VTA, in part, regulates binge-like ethanol consumption. Further, the lack of an effect observed

following site-specific TCS infusion suggests that OX2R signaling in the VTA may not significantly contribute to binge drinking behavior. Interestingly, although peripheral SB blunts binge-like consumption of a palatable reinforcer (Alcaraz-Iborra *et al*, 2014; Anderson *et al*, 2014; Olney *et al*, 2015), intra-VTA infusion of SB did not alter sucrose intake- suggesting that a distinct OX circuit is recruited for ethanol consumption versus a natural reward. What is more, the fact that these manipulations did not impact anxiety-like behavior suggests that OX1R antagonists protect against binge-like ethanol drinking independent of effects on anxiety-like behaviors. Together, these findings provide further support that OXR antagonists may be promising targets for treating alcohol use disorders (Khoo and Brown, 2014). Indeed, a pharmacotherapy that is effective in curbing ethanol drinking while leaving responding to natural reinforcers intact- as these data suggest OX1R antagonists are capable of- is certainly an appealing quality and should be further explored.

**Figure 3.1**: Cannula placements for the studies that examined pharmacological inhibition of OX1Rs in the VTA via SB on binge-like ethanol and sucrose (A) consumption, inhibition of OX2Rs in the VTA via TCS on binge-like ethanol consumption (B), the effect of intra-VTA SB administration on anxiety-like behavior (C), and the site-control study that infused SB dorsal to the VTA (D). Following histological assessment of cannula placement, it was determined that two subjects from the SB-VTA ethanol and sucrose studies, three from the TCS-VTA ethanol study, and three for the SB-VTA anxiety-like behavior studies were needed to be excluded due to poor cannula placement. Each filled circle represents the location of a cannula in the target region.

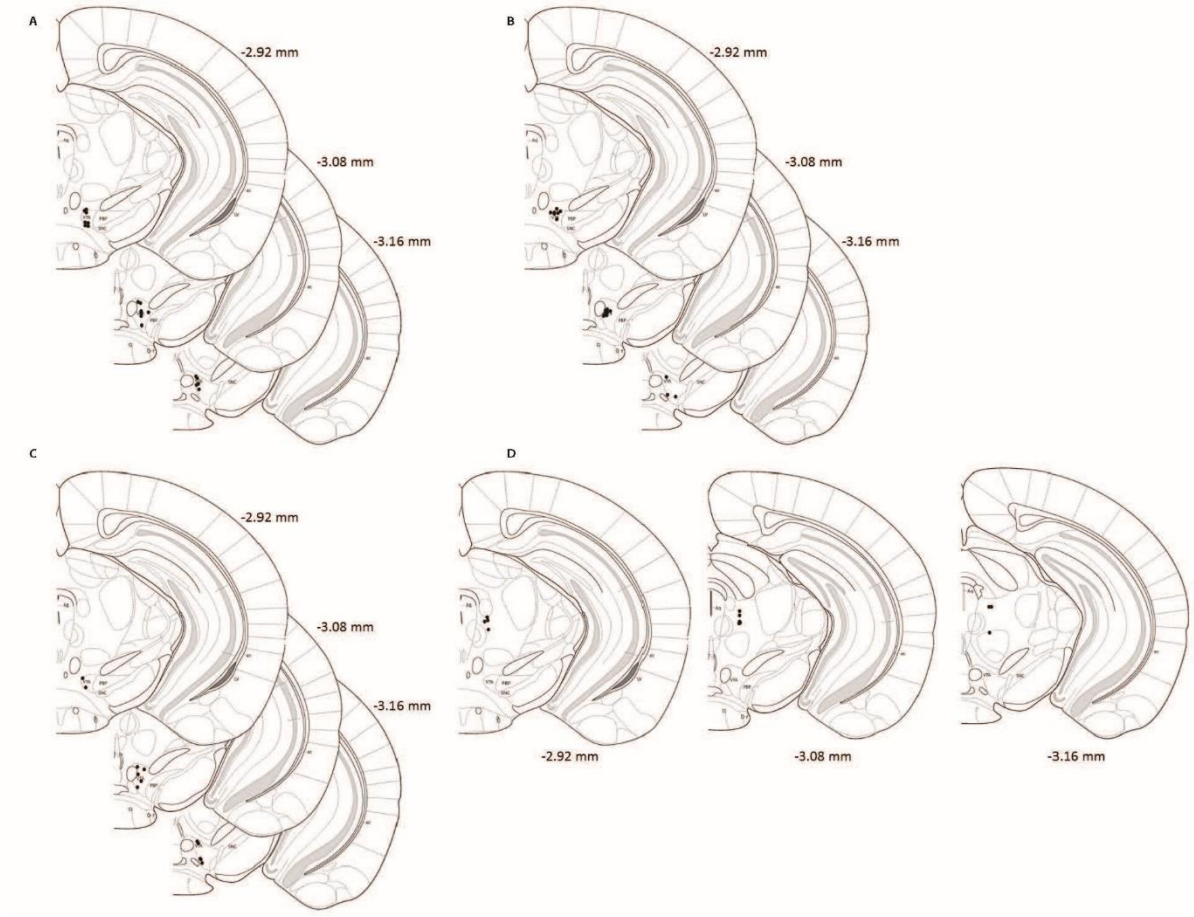


**Figure 3.2**: Inhibition of OX1Rs in the VTA selectively reduces binge-like ethanol consumption. Animals infused with SB directly into the VTA exhibited blunted binge-like ethanol consumption relative to vehicle-treated controls but only during the first hour of testing (A). Despite this reduction in consumption, no significant effect was observed in BECs as measured at the end of the two hour test period (B). Interestingly, intra-VTA infusion of SB did not significantly affect binge-like sucrose consumption at any point during the test (C) though a significant Time×Dose interaction revealed that vehicle-treated animals drank significantly more sucrose during the first hour of testing relative to the second. Importantly, this phenomenon was specific to the VTA treatment as SB infused directly dorsal to the VTA did not significantly impact binge-like ethanol drinking (D) or subsequent BECs (E) at the end of the two hour test period. We did, however, observe that animals drank significantly more ethanol during the second hour of testing relative to the first hour regardless of treatment group. * denotes that $p < 0.05$ relative to vehicle-treated animals during the first hour of testing. † signifies that $p < 0.05$ relative to vehicle-treated animals during the first hour of testing. # denotes that animals drank significantly more during the second hour of testing relative to the first hour regardless of treatment group. Dotted line in (B) delineates 80 mg/dl, the minimum BEC to constitute a binge episode. Data presented as Mean ± SEM.

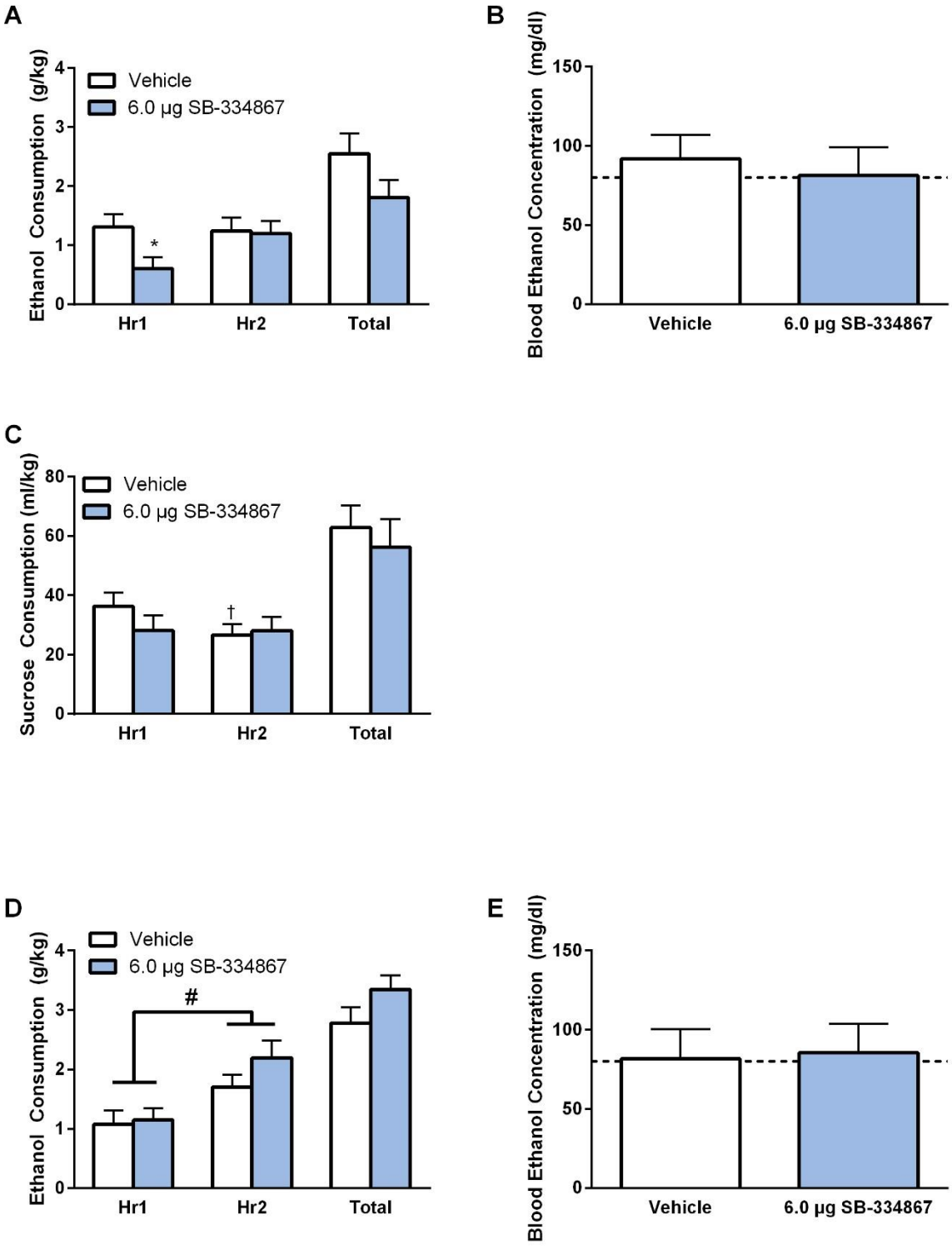

A
Ethanol Consumption (g/kg)
Vehicle
6.0 µg SB-334867
*
Hr1
Hr2
Total
B
Blood Ethanol Concentration (mg/dl)
Vehicle
6.0 µg SB-334867
C
Sucrose Consumption (ml/kg)
Vehicle
6.0 µg SB-334867
†
Hr1
Hr2
Total
D
Ethanol Consumption (g/kg)
Vehicle
6.0 µg SB-334867
#
Hr1
Hr2
Total
E
Blood Ethanol Concentration (mg/dl)
Vehicle
6.0 µg SB-334867

**Figure 3.3:** Inhibition of OX2Rs in the VTA does not significantly alter binge-like consumption. Infusion of TCS into the VTA does not significantly impact binge-like ethanol consumption at any point throughout the test period (A) nor does it significantly alter observed BEC levels (B). Dotted line in (B) delineates 80 mg/dl. Data presented as Mean ± SEM.

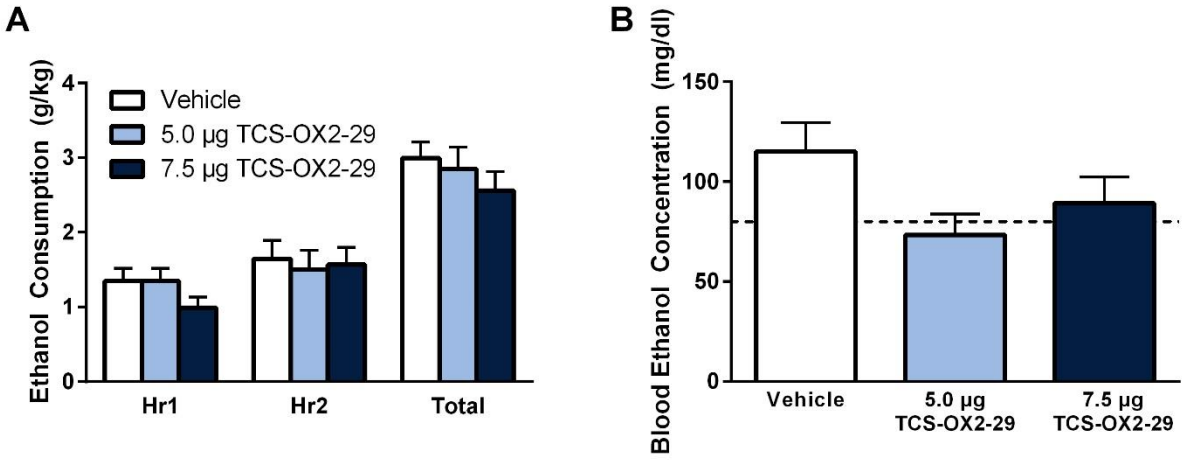


**Figure 3.4:** The effect on intra-VTA infusion of SB on anxiety-like behavior as measured via the elevated plus maze. Relative to treatment with vehicle, inhibition of OX1Rs within the VTA does not significantly alter time spent in the open area (A), the number of entries into the open area (B), or the number and duration of exploration behaviors (C; left and right axes, respectively). Data presented as Mean ± SEM.

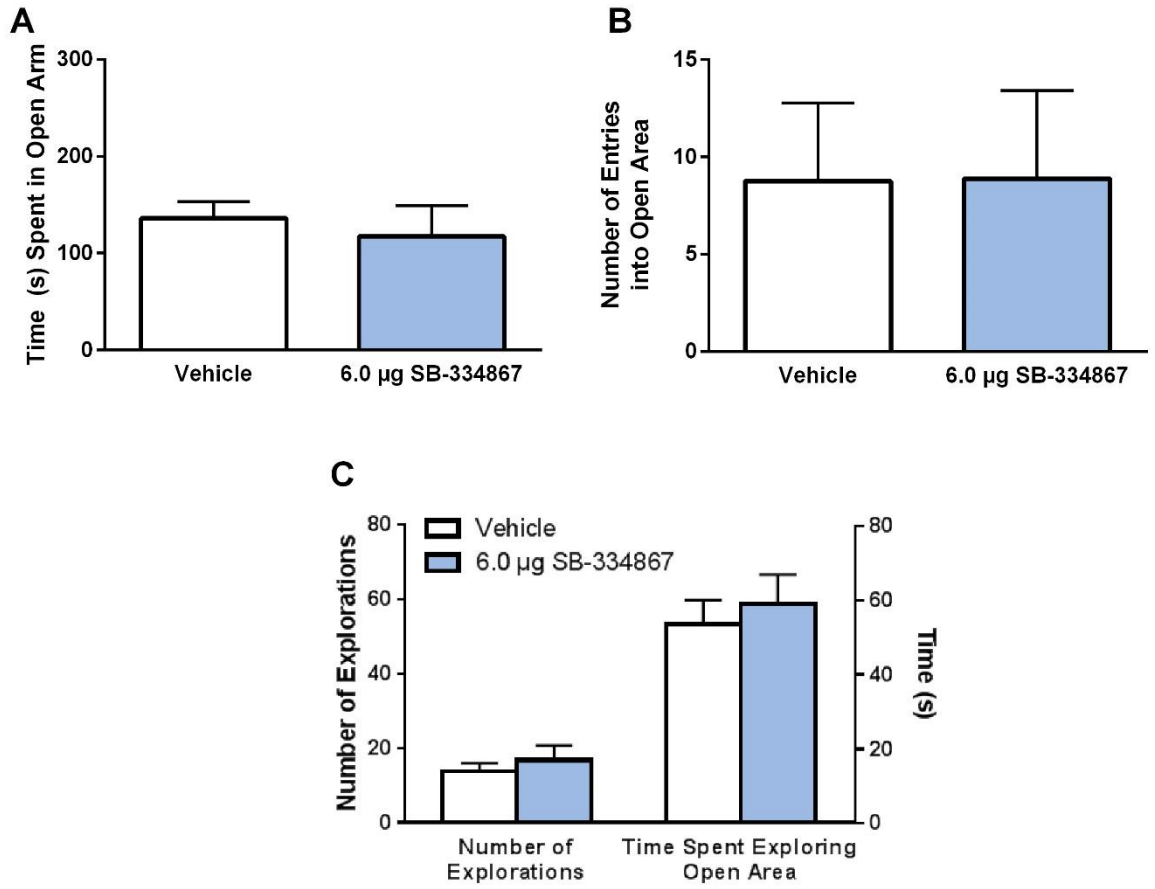
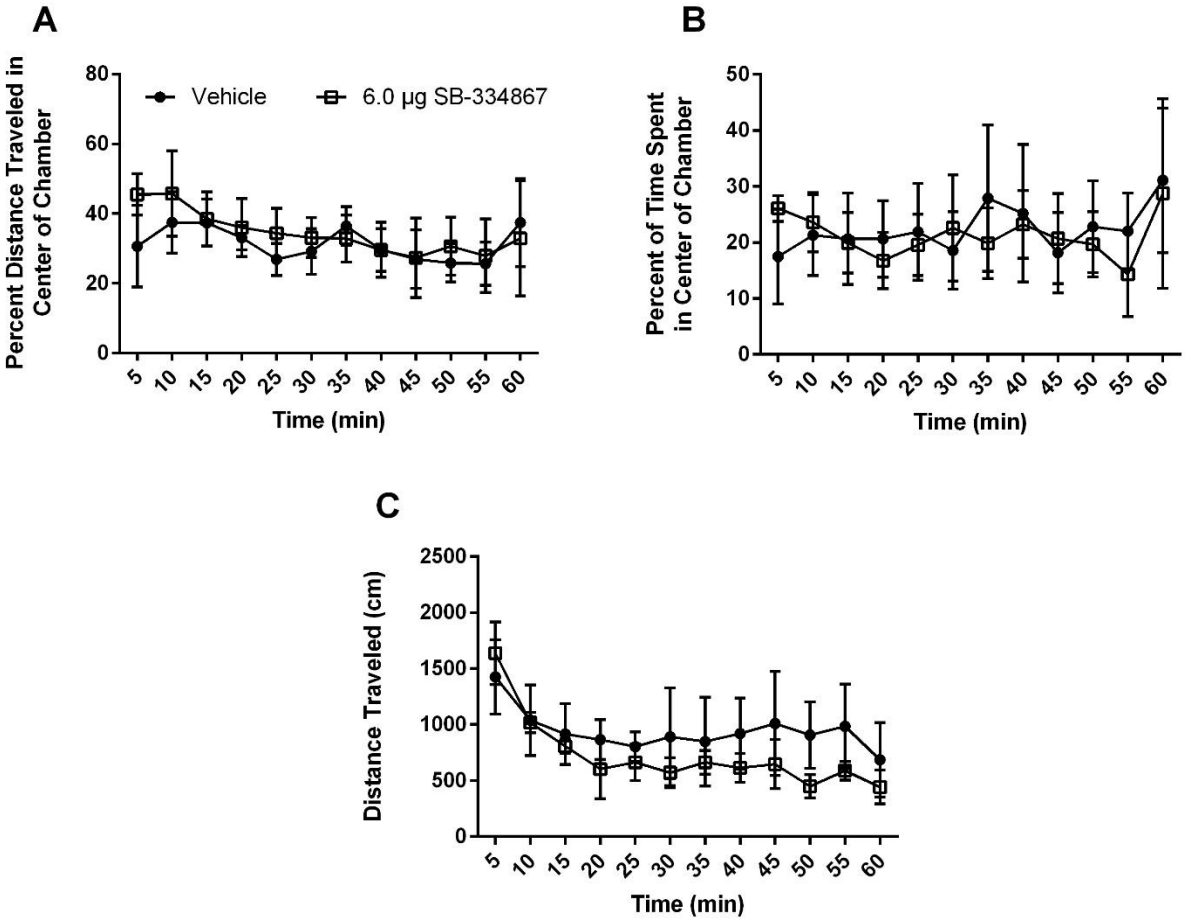


**Figure 3.5:** Inhibition of VTA OX1Rs via site-directed infusion of SB does not alter behavioral measures using the open-field locomotor assay. Relative to treatment with vehicle, suppression of activity onto the OX1R within the VTA does not affect measures of anxiety-like behavior: percent distance traveled in the center of the chamber (A) or the percent time spent in the center (B). Additionally, relative to vehicle-treated controls, animals infused with SB do not display detriments in locomotor behavior (C). Data presented as Mean ± SEM.



# CHAPTER 4

# PHARMACOLOGICAL INVESTIGATION OF THE ROLE OF OREXIN SIGNALING WITHIN STRESS-RELATED CIRCUITRY IN BINGE-LIKE ETHANOL DRINKING BEHAVIOR

## Introduction

Alcohol abuse is a serious health concern that affects individuals across a wide range of social strata (Naimi *et al*, 2003). Not surprisingly, alcohol abuse remains a leading cause of preventable death (Mokdad *et al*, 2004) and inflicts especially high social and economic costs on society (Bouchery *et al*, 2011; Sacks *et al*, 2015). As such, the development of effective treatment strategies that address this destructive behavior has the potential to have a major positive impact on society. Therefore, a better understanding of the biological mechanisms that modulate binge drinking behavior may be beneficial in facilitating the development of novel treatments for alcohol use disorders (AUDs).

One such mechanism that has been implicated in ethanol responding is the orexin (OX) system (see Lawrence, 2010 for review). Neurons that produce the OX peptides, orexin-A and -B, are highly localized within the hypothalamus yet project to numerous regions throughout the brain. These OX projection fibers release OX on to two G-protein coupled receptors, orexin-1 and orexin-2 receptors (OX1R and OX2R, respectively), to regulate a myriad of physiological functions including feeding (Sakurai *et al*, 1998), sleep (Chemelli *et al*, 1999), reward (Harris *et al*, 2005), and stress (Kuru *et al*, 2000). These latter two functions are of particular interest as

both positive- and negative-reinforcement are known to provide substantial impetus to drink ethanol (Eckardt *et al*, 1998; Ray *et al*, 2009).

The previous chapter demonstrated that inhibiting the OX1R in the ventral tegmental area (VTA) did not alter anxiety-like behavior; thus, blocking OX1R signaling, at least within in the VTA, modulates binge-like ethanol drinking independent of modulation of anxiety-like behavior. However, the VTA plays a relatively minor role in stress responding. In order to better elucidate the contribution of OX stress circuitry in binge drinking behavior, it is necessary to further investigate other brain regions that are more critically involved in stress and anxiety, such as the amygdala.

The amygdala is comprised of several subnuclei, among them the central nucleus of the amygdala (CeA) and basolateral amygdala (BLA), and is critically involved in emotional processing (see Janak and Tye, 2015 for review). Anatomical mapping studies that assessed immediate-early gene expression as a measure for neuronal activity have reported that both the BLA and CeA are engaged following ethanol administration in mice (Bachtell *et al*, 1999; Bachtell and Ryabinin, 2001); however, Pandey and colleagues (2008) observed signs of synaptic plasticity in the CeA, but not BLA, following acute ethanol administration- suggesting a more pivotal role for the CeA in neurobiological responses to ethanol. Further, the CeA has also been implicated in the expression of ethanol-induced anxiolysis (Pandey *et al*, 2008; Sharko *et al*, 2015).

What is more, previous investigations have reported rather dense orexinergic projections terminating in the CeA while relatively fewer fibers project to the BLA (Nambu *et al*, 1999; Peyron *et al*, 1998; Schmitt *et al*, 2012). Indeed, application of both orexin-A and -B results in a

robust depolarization of CeA neurons *in vitro* (Bisetti *et al*, 2006; Winsky-Sommerer *et al*, 2004) and central administration of OX *in vivo* engages neurons within the CeA (Hata *et al*, 2011; Sakamoto *et al*, 2004). Taken together, these findings suggest that OX input onto the CeA may modulate binge-like ethanol drinking secondarily through modulation of stress responses, consistent with the idea that ethanol consumption may be reinforced, in part, via stress reduction.

In addition to modulating stress processing, findings from multiple lines of research implicate the CeA as a key modulator of reward processing. In fact, the CeA is considered part of the mesolimbic reward circuit and sends projections to both the VTA (Zahm *et al*, 1999) and nucleus accumbens (Vertes *et al*, 2012). Indeed, optogentic stimulation of neurons within the CeA greatly enhances motivation towards a reward-paired cue (Robinson *et al*, 2014). Moreover, the actions of a litany of other peptides, such as neurotensin (László *et al*, 2010), oxytocin (László *et al*, 2016), and substance P (Kertes *et al*, 2009), within the CeA have also been demonstrated to modulate reward-related behaviors. Furthermore, it has previously been demonstrated that administration of a positive modulator of AMPA receptors in the CeA increased operant responding for ethanol (Cannady *et al*, 2016). Together, these data indicate that the CeA is involved in reward-related processing in addition to stress responding.

The goal present chapter was to characterize the contribution OXR signaling within the CeA in modulating binge-like ethanol drinking. To ascertain whether modulation of binge-like ethanol drinking is associated with modulation of anxiety-like behavior, we also tested the effect of intra-CeA OXR inhibition on anxiety-like behaviors. As predicted, inhibiting OX signaling within the CeA successfully attenuated binge-like ethanol drinking. More specifically, local infusions of either an OX1R or OX2R antagonist into the CeA reduced ethanol, but not sucrose, intake. Additionally, we also observed that similar inhibition of OX1Rs in the neighboring BLA

did not affect binge-like ethanol or sucrose drinking. Inconsistent with our predictions, however, was the fact that blocking OX1R signaling in the CeA did not impact anxiety-like behaviors in either the elevated zero maze or open-field locomotor chamber. Together, these findings indicate that OXR signaling in the CeA participates in binge ethanol drinking and suggests that this region may modulate this behavior by altering the positive-reinforcing, rather than negative-reinforcing, consequences of ethanol consumption.

**Methods**

*Animals*

Male C57BL/6J mice (C57; Jackson Laboratories, Bar Harbor, ME), aged 6-7 weeks and weighing 20-25 g upon arrival, were used in each of the following studies. Mice were individually housed in plastic cages located in a vivarium with an ambient temperature of approximately 22ºC and a reverse light/dark cycle with lights off at 8:30 am. All animals had *ad libitum* access to food and water except when specified below. All procedures used were in accordance with the National Institute of Health guidelines and were approved by the University of North Carolina Institutional Animal Care and Use Committee.

*Cannulation Surgery and Infusion Procedures*

Prior to testing, mice were bilaterally cannulated targeting the CeA (AP: -1.06 mm, ML: ±2.35 mm, DV: -4.50 mm). An additional cohort of animals was bilaterally cannulated targeting the BLA (AP: -1.22 mm, ML: ±3.01 mm, DV: -4.74 mm) as a region-specific control. Mice were given one week to recover from surgery before testing. Cannula placement for each animal was verified histologically and animals in which the cannulas were not in the target area were excluded from the statistical analysis (see Figure 4.1).

On test days, mice were infused with either the selective OX1R antagonist, SB-334867 (0.0 or 6.0 µg; Tocris Bioscience, Bristol, UK), or the selective OX2R antagonist, TCS-OX2-29 (0.0 or 7.5 µg; Tocris Bioscience, Bristol, UK). These compounds are 50- and 250-fold, respectively, selective for their respective OXR target over the other subtype (Hirose *et al*, 2003; Porter *et al*, 2001; Smart *et al*, 2001). The decision to use only the higher dose (7.5 µg) of TCS was due to the fact that we did not observe any significant effects of the lower dose (5.0 µg) from our previous investigations in the VTA (see Chapter 3). Both compounds were dissolved in dimethyl sulfoxide (DMSO) to reach the desired concentration. All infusions were administered in a volume of 0.3 µl per side and were delivered over the course of one minute using an automated syringe pump (Harvard Apparatus, Holliston, MA). Injectors were left in the cannulae for an extra minute before removal to ensure adequate diffusion of the compound and to prevent reflux up the cannula tract. Drug infusions for all studies were performed 30 min prior to the start of the behavioral test.

*Drinking in the Dark*

A cohort of 15 C57s was used to assess the effect of pharmacological inhibition of CeA OX1Rs on binge-like consumption. However, the cannula of one animal became clogged following ethanol DID testing; therefore, only 14 C57s were used in the intra-CeA sucrose DID test. This same group of mice was also used to similarly assess inhibition of CeA OX2Rs. Additionally, another cohort of 13 mice was used to investigate the site-specificity of this effect by assessing binge-like ethanol consumption following local infusion into the BLA. The DID procedure is a commonly used animal model of binge-like ethanol drinking that promotes high levels of consumption and generates physiologically relevant blood ethanol concentrations (BECs) of 80 mg/dl or greater (Rhodes *et al*, 2005, 2007). On days 1-3, standard water bottles

were removed three hours into the dark cycle and the animals were given access to a single test bottle containing ethanol (20% v/v) or sucrose (3% w/v) solutions for two hours. Binge-like intake was assessed on the fourth day in which test bottles were measured hourly in order to examine the effect of the drug on consumption over time. Immediately after test bottles were removed on the fourth day of ethanol testing, tail-blood samples were taken from each animal and processed to determine BEC. As with the previous chapter, a shortened test period was chosen in order to better capture the transient effects of the compounds. In order to increase power during statistical analysis, a Latin-square design was used such that each animal received all doses of the drug over repeated trials. Mice were given three days of rest between subsequent 4-day DID sessions in order to avoid carryover effects of the drug.

*Elevated Zero Maze*

The same mice used to investigate the contribution of OX1R signaling on binge-like consumption were also used assess the effect of pharmacological inhibition of CeA OX1Rs on anxiety-like behavior using a five minute test on the elevated zero maze (Med Associates, Inc., St. Albans, VT). The elevated zero maze is a common tool used among investigators in order to assess anxiety-like behavior. Here, anxiolytic drugs increase the amount of time spent in the open area while anxiogenic drugs increase the amount of time spend in the closed areas (Shepherd *et al*, 1994). One hour before testing, animals were brought into a dark room that housed the elevated zero maze. Approximately three hours into the dark cycle, testing on the elevated zero maze began by placing the animal in the open area of the maze. Each session was recorded using a camcorder placed above the apparatus and was scored by an investigator blind to the animal's group assignment who recorded the time (s) spent in the open area as well as the number of entries into the open and closed area. The animal was considered to have entered the

open area when all four paws left the closed area. Open area time was considered terminated once all of the animal's paws entered the closed area. We also assessed the number of instances and time (s) spent "exploring" the open area from the closed area. Exploring behavior was defined as each time the animal, while still in the closed area, extended its head beyond the ears into the open area. Exploring behavior ceased when the animal either entered the open area or retreated back into the closed area. Animals were returned to their homecage immediately after the conclusion of the test session. Unlike DID testing, a Latin-square design was not used in order to avoid previous experience with the apparatus confounding the animals' behavior. Treatment groups were equated based on body weight and each animal received a single, bilateral infusion of either 0.0 or 6.0 µg of SB.

*Open-Field Locomotor Activity*

The same group of animals from the previous elevated zero maze test was used to assess the effect of pharmacological inhibition of CeA OX1Rs on locomotor activity; however, one animal's cannula became clogged in the interim time between tests and was unable to be infused. Thus, only 14 C57s were used. In addition to assessing general locomotor activity in the animals, this test is capable of measuring anxiety-like behavior as well. Similar to the elevated zero maze, treatment with anxiolytic compounds generally causes the animal to increase the amount of time spent in the center of the open-field chamber (Choleris *et al*, 2001). Testing in the open-field locomotor chambers occurred 48 h after testing in the elevated zero maze. One hour before testing, animals were brought into a dark room adjacent to the room that housed the locomotor chambers. Three hours into the dark cycle, animals were placed in a 16.5 x 16.5 $in^2$ open-field locomotor chamber (Accuscan Instruments, Columbus, OH) and locomotor activity was recorded in five minute bins for one hour using VersaMax software (Omnitech Electronics,

Columbus, OH). This software was able to record total distance traveled (cm) as well as the time (s) spent and distance traveled (cm) in the center or margin of the chamber. Using these measures, we calculated separate variables for the percent time spent in the center of the chamber ([time spent in the center ÷ (time spent in the center + time spent in the margins)] × 100) as well as the percent distance traveled in the center of the chamber ([distance traveled in the center ÷ (distance traveled in the center + distance traveled in along the margins)] × 100). Mice were placed back into their homecages after the one hour test period. Like the elevated plus maze, a Latin-square design was not used for the locomotor test. Animals received a single, bilateral infusion of either 0.0 or 6.0 μg of SB. Each animal received the alternate drug treatment based on its assigned drug condition during the previous elevated zero maze test.

*Data Analysis*

A repeated-measures ANOVA was used to assess hourly binge-like consumption with both time (hour 1 and hour 2) and dose (0.0 or 6.0 μg for SB; 0.0 or 7.5 μg for TCS) being within-subject variables. Additionally, BEC and total binge consumption across the two-hour test period was assessed using separate repeated-measures ANOVAs with dose (0.0 or 6.0 μg for SB; 0.0 or 7.5 μg for TCS) as the within-subject variable. We also included drug order as a between-subjects variable in these analyses to ensure the order in which the animals were presented the drug did not have any confounding effects on drinking behavior. Importantly, due to the relatively short half-life and hyper-transient nature of the compounds (Mould *et al*, 2014; Porter *et al*, 2001), the effects of these compounds can be rather short-lived (Olney *et al*, 2015). Thus, planned comparisons were used to assess binge-like consumption of each drug group relative to its respective vehicle during the first hour of testing in order to better capture the short-lived effect of the compound.

Additionally, separate univariate ANOVAs used dose of SB (0.0 or 6.0 µg) to analyze the time spent in the open area, the number of entries into the open area, time spent exploring the open area, and frequency of exploratory behavior in the elevated plus maze paradigm. Similarly, separate repeated-measures ANOVAs were used to assess behavior in the open-field locomotor tests with dose of SB (0.0 or 6.0 µg) as a between-subjects variable and total distance traveled, percent distance traveled in the center, and percent time spent in the center across the twelve 5-min bins assessed as within-subject variables. Tukey's LSD *post-hoc* tests and Bonferroni corrections were employed throughout the analyses when applicable.

**Results**

Repeated measures ANOVA revealed that binge-like ethanol drinking did not change over the course of the two hour test period (Figure 4.2A; Main effect of Time: $F_{(1,13)} = 2.077$, p = 0.173). Although there was no main effect of dose ($F_{(1,13)} = 2.242$, p = 0.158), there was a trend for drinking levels to change over time as a function of treatment, but this effect did not reach significance (Time×Dose Interaction: $F_{(1,13)} = 2.334$, $p = 0.056$); however, planned comparisons at the first hour of testing revealed that intra-CeA treatment with SB significantly reduced drinking levels relative to vehicle-treated controls (planned comparison: ($t_{(14)} = 2.961$, $p = 0.010$). Similarly, our analyses did not reveal an effect of treatment on total drinking across the two hour test period ($F_{(1,13)} = 2.242$, $p = 0.158$) nor did it impact BECs Figure 4.2B; $F_{(1,11)} = 0.149$, $p = 0.707$). Importantly, whether the animal was treated with SB or vehicle first did not affect binge-like ethanol drinking ($F_{(1,13)} = 0.135$, $p = 0.719$) or BECs ($F_{(1,11)} = 0.001$, $p = 0.982$).

This effect was observed to be specific to ethanol as similar treatment did not alter hourly sucrose consumption (Main effect of time: $F_{(1,13)} = 1.707$, $p = 0.214$; Main effect of Dose: $F_{(1,13)}$

= 1.957, $p = 0.185$; Time×Dose Interaction: $F_{(1,13)} = 0.547$, $p = 0.473$; planned comparison at the first hour: $t_{(14)} = 2.035$, $p = 0.061$) or total consumption across the two-hour test period ($F_{(1,13)} = 1.957$, $p = 0.185$; Figure 4.2C). Moreover, drug order did not impact binge-like sucrose consumption ($F_{(1,13)} = 0.503$, $p = 0.491$).

Not only was this effect reinforcer-specific, our analyses also revealed this effect to be specific to the CeA as intra-BLA infusion of SB did not affect binge-like ethanol drinking (Figure 4.2D; Main effect of Dose: $F_{(1,11)} = 0.088$, $p = 0.772$; Time×Dose Interaction: $F_{(1,11)} = 0.002$, $p = 0.962$; planned comparison at the first hour: $t_{(12)} = 0.134$, $p = 0.896$)- although there was a significant effect that indicated that animals drank significantly more ethanol during the second hour of testing relative to the first regardless of drug treatment (Main effect of Time: $F_{(1,1)} = 7.522$, $p = 0.019$). Moreover, there was no significant effect of SB across the total two hour test period ($F_{(1,11)} = 0.088$, $p = 0.772$) nor was there an effect on BECs (Figure 4.2E; $F_{(1,11)} = 0.795$, $p = 0.392$). The order in which the animals received the drug did not alter drinking behavior ($F_{(1,11)} = 0.045$, $p = 0.836$) or BECs ($F_{(1,11)} = 0.503$, $p = 0.493$).

With the OX2R antagonist, we did not observe any main effects of TCS to alter binge-like ethanol drinking over time (Figure 4.3A; Main effect of Dose: $F_{(1,13)} = 2.344$, $p = 0.150$; Main effect of Time: $F_{(1,13)} = 0.833$, $p = 0.378$); however, we did observe a significant interaction effect (Time×Dose Interaction: $F_{(1,13)} = 8.980$, $p = 0.010$). Interestingly, further probing revealed that this effect was driven by the fact that vehicle treated animals drank significantly more ethanol during the second hour of testing relative to the first hour ($t_{(14)} = -3.076$, $p = 0.008$). Further probing of this effect indicated that vehicle treated animals consumed significantly more ethanol relative to TCS treated animals during the second hour of testing ($t_{(14)} = 3.190$, $p = 0.007$). Notably, no difference was observed between the two treatment groups

during the first hour of testing ($t_{(14)}$ = -1.284, $p$ = 0.220). No effect was observed across the total two hour test period ($F_{(1,13)}$ = 2.330, $p$ = 0.151) nor was there an effect on BECs (Figure 4.3B; $F_{(1,13)}$ = 0.631, $p$ = 0.445). Treatment order did not affect ethanol drinking ($F_{(1,13)}$ = 150, $p$ = 0.705) or BECs ($F_{(1,13)}$ = 4.148, $p$ = 0.069).

To fully characterize the role of OX2Rs in the CeA, we also investigated the effects of direct infusions of TCS on binge-like sucrose consumption as well. Our omnibus analyses yielded no significant effect of intra-CeA infusion of TCS on any measure (Figure 4.3C; Main effect of Time: $F_{(1,12)}$ = 0.046, $p$ = 0.834; Main effect of Dose: $F_{(1,12)}$ = 0.462, $p$ = 0.510; Time×Dose Interaction: $F_{(1,12)}$ = 0.872, $p$ = 0.369; planned comparison at the first hour: $t_{(13)}$ = -0.709, $p$ = 0.491; total drinking across the full two hours: $F_{(1,12)}$ = 0.462, $p$ = 0.510; drug order: $F_{(1,12)}$ = 0.277, $p$ = 0.608).

What is more, the effect of inhibiting OX1Rs within the CeA was found not likely to be secondary to relief from a stressful state. Specifically, intra-CeA treatment with SB did not significantly alter the amount of time the animal spent in the open area of the elevated zero maze (Figure 4.4A; $F_{(1,13)}$ = 0.230, $p$ = 0.639) or the number of entries into the open area (Figure 4.4B; $F_{(1,13)}$ = 0.007, $p$ = 0.933). Moreover, such treatment did not affect the number of instances (Figure 4.4D; $F_{(1,13)}$ = 0.738, $p$ = 0.406) or time ($F_{(1,13)}$ = 0.166, $p$ = 0.690) spent exploring the open area.

Similar treatment with SB did not alter the proportion of distance traveled in the center of the chamber (Figure 4.5A; Main effect of Time: $F_{(11,132)}$ = 1.484, $p$ = 0.145; Main effect of Dose: $F_{(1,12)}$ = 0.049, $p$ = 0.828; Time×Dose Interaction: $F_{(11,132)}$ = 0.453, $p$ = 0.928) or proportion of time spent in the center (Figure 4.5B; Main effect of Time: $F_{(11,132)}$ = 0.872, $p$ =

0.454; Main effect of Dose: $F_{(1,12)} = 0.030$, $p = 0.866$; Time×Dose Interaction: $F_{(11,132)} = 0.643$, $p = 0.573$). Importantly, inhibition of OX1Rs within the CeA did not significantly impact general locomotor activity (Figure 4.5C; Main effect of Dose: $F_{(1,12)} = 1.040$, $p = 0.328$; Time×Dose Interaction: $F_{(11,132)} = 1.236$, $p = 0.270$). There was, however, a significant effect of time (Main effect of Time: $F_{(11,132)} = 73.347$, $p < 0.001$). Further probing of this effect with Tukey's LSD indicated that the animals tended to display greater activity towards the beginning of the test with the significant differences at the following time points: 5 min > all other time points ($p$'s < 0.001), 10 min > all subsequent time points ($p$'s < 0.001), 15 min > all subsequent time points after 20 min ($p$'s ≤ 0.002), 20 min > 40 min ($p = 0.003$), 35 min > 60 min ($p = 0.002$).

**Discussion**

The previous chapter identified the VTA as a brain region receiving OX input that helps regulate binge-like ethanol drinking behavior. Results from the current chapter expand upon these findings by implicating the CeA as a target of OX input that modulates binge-like ethanol consumption as well. Through our series of experiments, we demonstrated that OX1R- and possibly OX2R- signaling within the CeA selectively reduced binge-like ethanol, but not sucrose, intake. Moreover, such an effect was specific to the CeA as intra-BLA infusion of the OX1R antagonist, SB, did not attenuate binge-like ethanol drinking. Interestingly, we demonstrated that local inhibition of OX1R signaling in the CeA did not alter anxiety-like behavior as measured by the elevated zero maze and open-field locomotor test. These effects were not due to alterations in sleep and arousal as intra-CeA infusion of SB did not affect general locomotor activity. Together, these findings suggest that OXR signaling within the CeA

participates in modulating binge-like ethanol drinking independent of OX circuitry involved in stress responding.

The data from the current experiments provides convincing support that the OX1Rs within the CeA contribute to binge-like ethanol drinking. Additionally, our findings suggest that OX2Rs may participate in this behavior as well. Although we observed that TCS treated animals consumed less ethanol relative to vehicle treated animals during the second hour of our ethanol DID test (Figure 4.3A), one should interpret these effects cautiously. Indeed, we also observed an odd effect that vehicle treated animals displayed elevated ethanol consumption during the second hour of testing relative to the first. Thus, treatment with TCS may not have reduced binge-like drinking behavior, *per se*, but rather blocked an increase in drinking behavior that occurred during the second hour of testing. Notably, we did not assess OX2R inhibition in the BLA nor did we explore anxiety-like behavior following infusion of TCS as the effects of the OX2R on binge-like ethanol drinking were less conclusive than those regarding the OX1R.

Together with our findings from the previous chapter, these data prompt for a more thorough examination of the subtle nuances of OX2R signaling in binge drinking behavior. For example, such an investigation could infuse a virus packaged with small interfering RNA (siRNA) that disrupts the production of the OX2R directly into the CeA. If OX2Rs within the CeA play a significant role in binge drinking then one would predict that knockdown of the OX2Rs would cause the animal not to engage in binge-like ethanol drinking in a DID model. Taking this concept a step further, one could use a similar siRNA strategy to knockdown the OX1R while using a separate virus to overexpress the OX2R. In this case, elimination of the OX1R would cause a reduction in binge-like ethanol drinking; however, if the OX2R contributes

to binge-like ethanol drinking then overexpressing the OX2R would rescue this phenotype such that the animal would once again engage in binge drinking.

Additionally, we observed that infusion of OXR antagonists within the CeA attenuated binge-like ethanol drinking but did not affect sucrose consumption. This is rather surprising as multiple sources have demonstrated that peripheral OXR antagonists disrupt binge-like ethanol consumption as well as that of caloric (sucrose) and non-caloric (saccharin) reinforcers (Alcaraz-Iborra *et al*, 2014; Anderson *et al*, 2014; Olney *et al*, 2015), which indicates that the OX system is involved in regulating responding to general, salient reinforcers whether it be ethanol or natural rewards (e.g. sucrose). However, findings from the current and previous chapters as well as other studies (Schneider *et al*, 2007; Srinivasan *et al*, 2012) have reported that OX signaling within specific brain regions does not participate in responding to natural reinforcers. In fact, Barson and colleagues (2014) discovered that distinct anatomical subregions within the same structure may be differentially involved in the consumption of ethanol versus that of a natural reward. In that study, the authors demonstrated that orexin-A infused directly into the anterior portion of the paraventricular nucleus of the thalamus (PVT) augmented ethanol, but not sucrose, drinking; however, similar infusion of the peptide into the posterior PVT elevated sucrose, but not ethanol, intake. As a whole, these findings support the idea that the OX circuitry that regulates ethanol responding may be largely independent of that which controls responding to natural rewards.

As with the findings from our VTA studies in the previous chapter, we did not observe any alterations in anxiety-like behavior following inhibition of OX1R signaling within the CeA as measured by the elevated plus maze and the open-field locomotor chamber, which indicates that OX signaling within the CeA does not modulate binge-like ethanol drinking by altering

stress responding. Interestingly these data suggest that inhibiting OX1R signaling in this region depresses reward processing, however, additional tests such as operant tasks are required before any definitive conclusions can be drawn.

The results from current chapter, to the best of our knowledge, provide the first evidence that OX signaling within the CeA modulates ethanol drinking behavior. Further, these data indicate that this circuit (1) does not include the BLA, (2) does not regulate consumption of natural rewards, and (3) does not engage stress-related circuitry. Together, these findings further establish the OX system as an attractive target for treating alcohol use disorders.

**Figure 4.1**: Cannula placements for the studies that examined pharmacological inhibition of OX1Rs in the CeA via SB on binge-like ethanol and sucrose consumption as well as for the tests measuring anxiety-like behaviors (A), inhibition of OX2Rs in the CeA via TCS on binge-like ethanol and sucrose consumption (B), and the site-control study that infused SB into the BLA (C). Following histological assessment of cannula placement, it was determined that four subjects from the SB-CeA studies, two from the TCS-VTA studies, and two from the SB-BLA studies were needed to be excluded due to poor cannula placement. Each filled circle represents the location of a cannula in the target region.

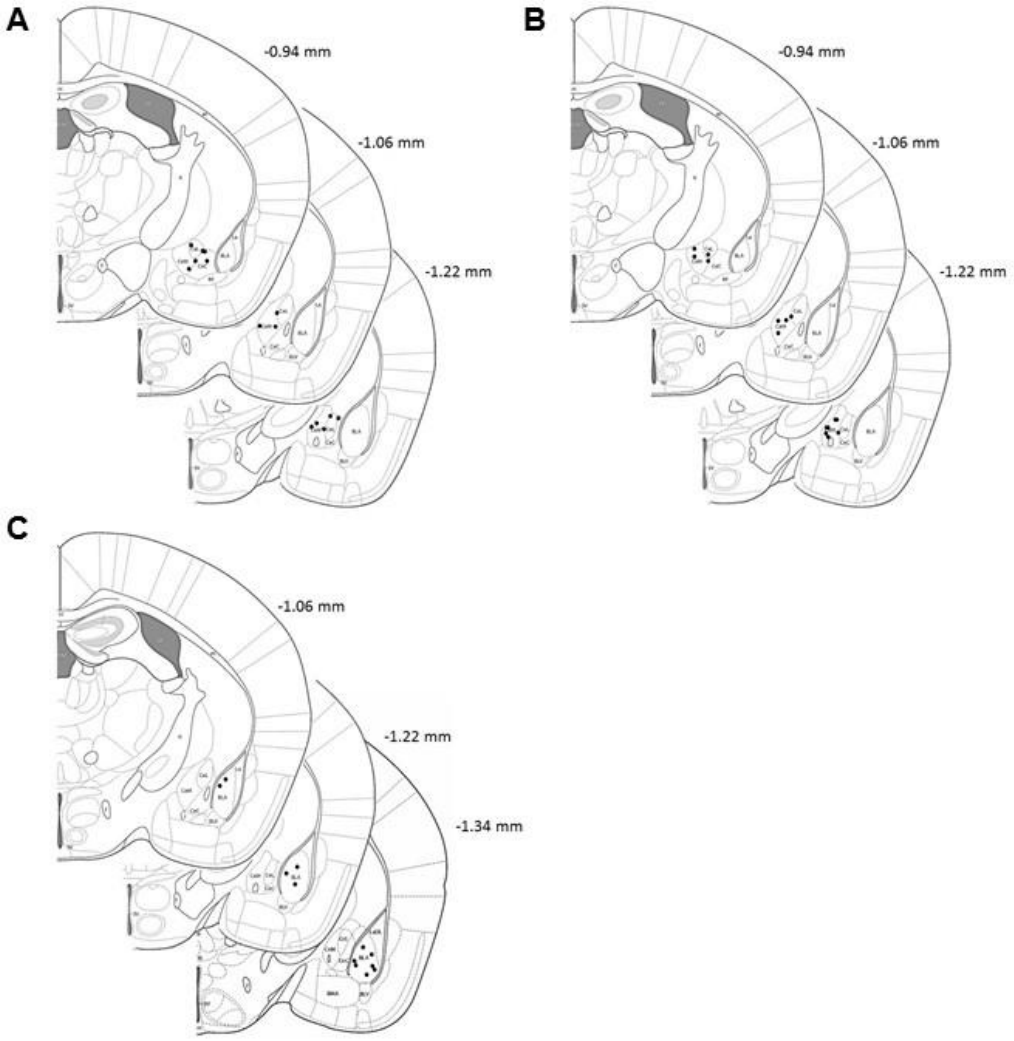


**Figure 4.2**: Inhibition of OX1Rs in the CeA selectively reduces binge-like ethanol consumption. Animals infused with SB directly into the CeA exhibited blunted binge-like ethanol consumption relative to vehicle-treated controls but only during the first hour of testing (A). Despite this reduction in consumption, no significant effect was observed in BECs as measured at the end of the two hour test period (B). Interestingly, intra-CeA infusion of SB did not significantly affect binge-like sucrose consumption at any point during the test (C). Importantly, this phenomenon was specific to the CeA treatment as local infusion of SB into the BLA did not significantly impact binge-like ethanol drinking (D)- although animals did drink significantly more ethanol during the second hour of testing relative to the first, regardless of treatment condition. Moreover, intra-BLA SB did not affect subsequent BECs (E) at the end of the two hour test period. Data presented as mean ± SEM. * denotes that $p < 0.05$ relative to vehicle-treated animals during the first hour of testing. # denotes that animals drank significantly more during the second hour of testing relative to the first hour regardless of treatment group. Dotted line in (B) delineates 80 mg/dl, the minimum BEC to constitute a binge episode. Data presented as Mean ± SEM.

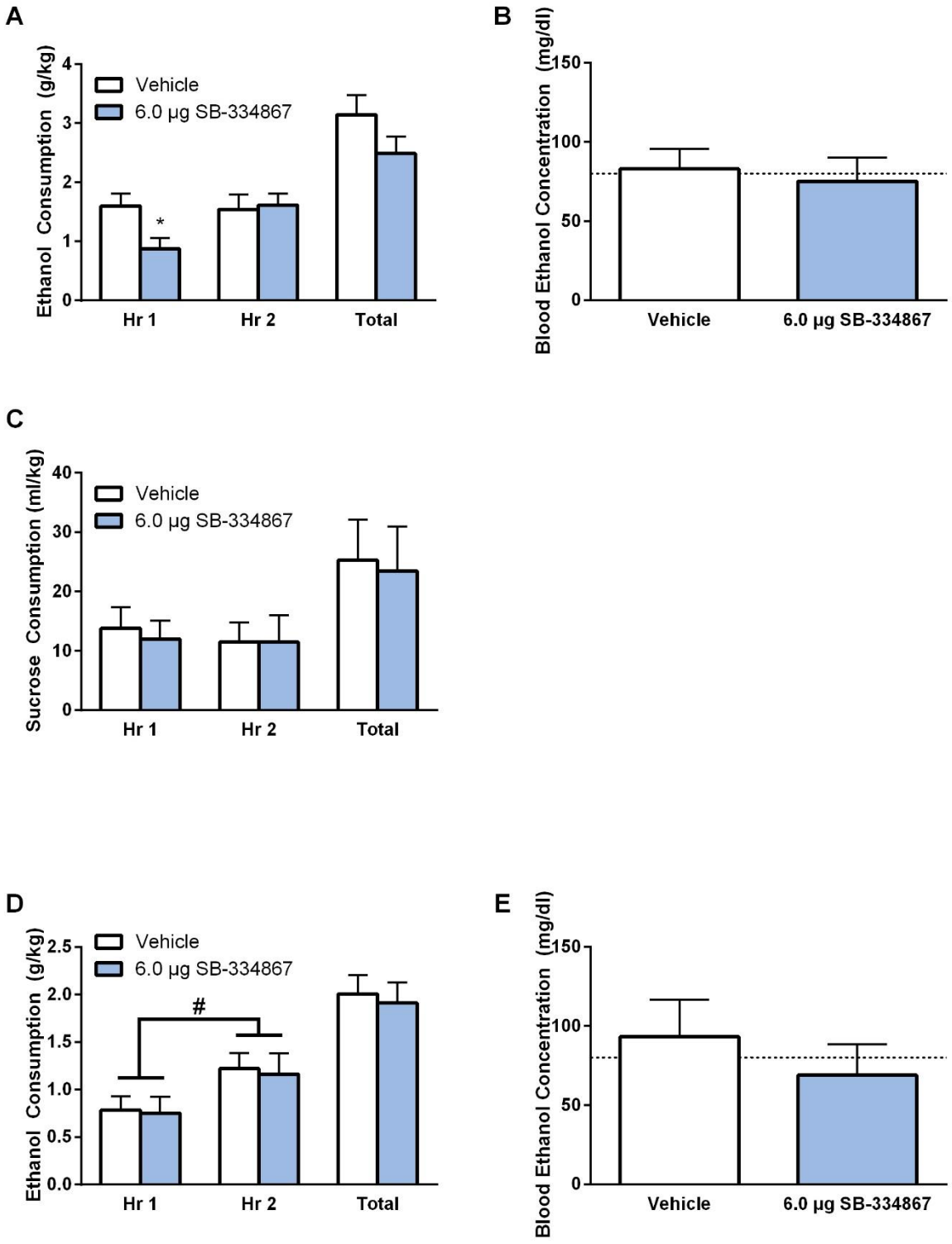

A
Ethanol Consumption (g/kg)
Vehicle
6.0 µg SB-334867
*
Hr 1
Hr 2
Total
B
Blood Ethanol Concentration (mg/dl)
Vehicle
6.0 µg SB-334867
C
Sucrose Consumption (ml/kg)
Vehicle
6.0 µg SB-334867
Hr 1
Hr 2
Total
D
Ethanol Consumption (g/kg)
Vehicle
6.0 µg SB-334867
#
Hr 1
Hr 2
Total
E
Blood Ethanol Concentration (mg/dl)
Vehicle
6.0 µg SB-334867

**Figure 4.3:** Inhibition of CeA OX2Rs impacts binge-like consumption. Vehicle treated animals displayed an enhancement in binge-like ethanol drinking during the second hour of testing relative to the first (A) and infusion of TCS into the CeA blocked this behavior. However, no changes in BECs were observed as a function of treatment with TCS (B) nor did it impact binge-like sucrose intake (C). Data presented as mean ± SEM. * denotes that $p < 0.05$ relative to vehicle treated animals during the first hour of testing. † signifies that $p < 0.05$ relative to TCS treated animals at the same time point. Dotted line in (B) delineates 80 mg/dl. Data presented as Mean ± SEM.

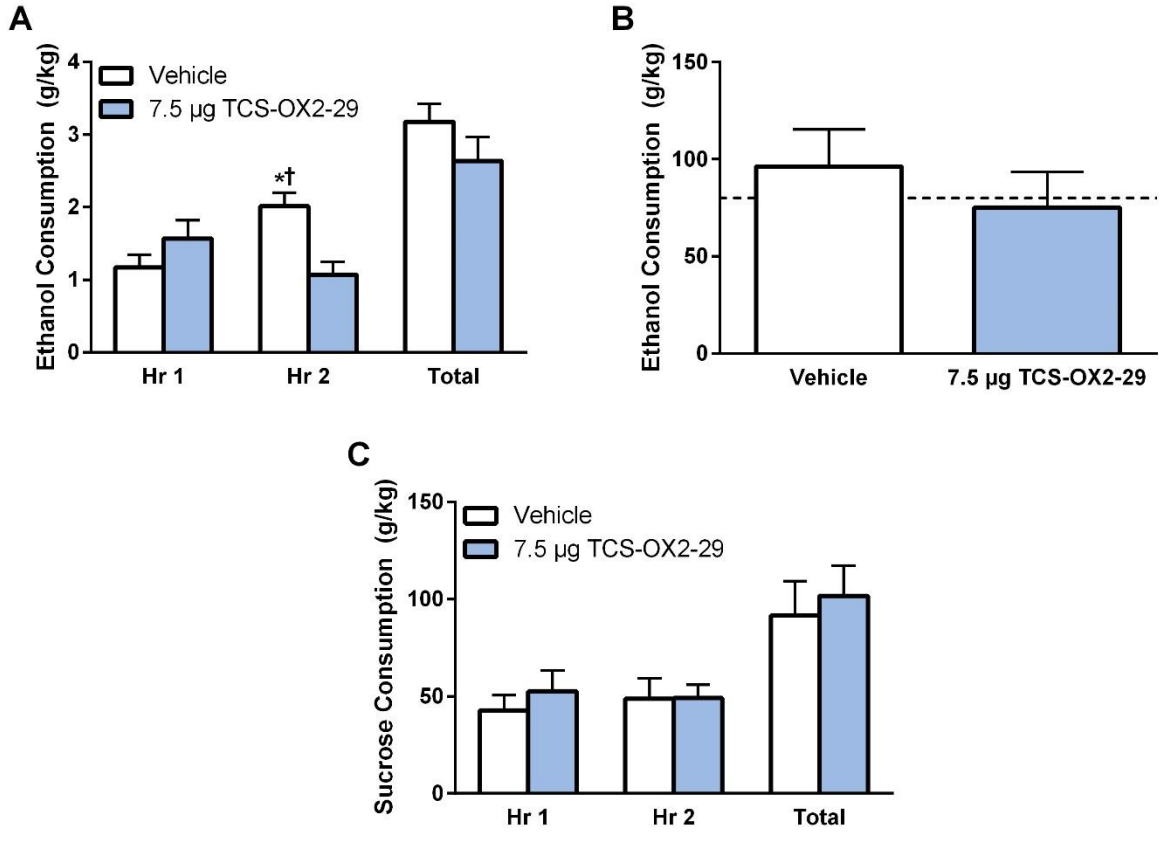


**Figure 4.4:** The effect on intra-CeA infusion of SB on anxiety-like behavior as measured via the elevated plus maze. Relative to treatment with vehicle, inhibition of OX1Rs within the VTA does not significantly alter time spent in the open area (A), the number of entries into the open area (B), or the number and duration of exploration behaviors (C; left and right axes, respectively). Data presented as Mean ± SEM.

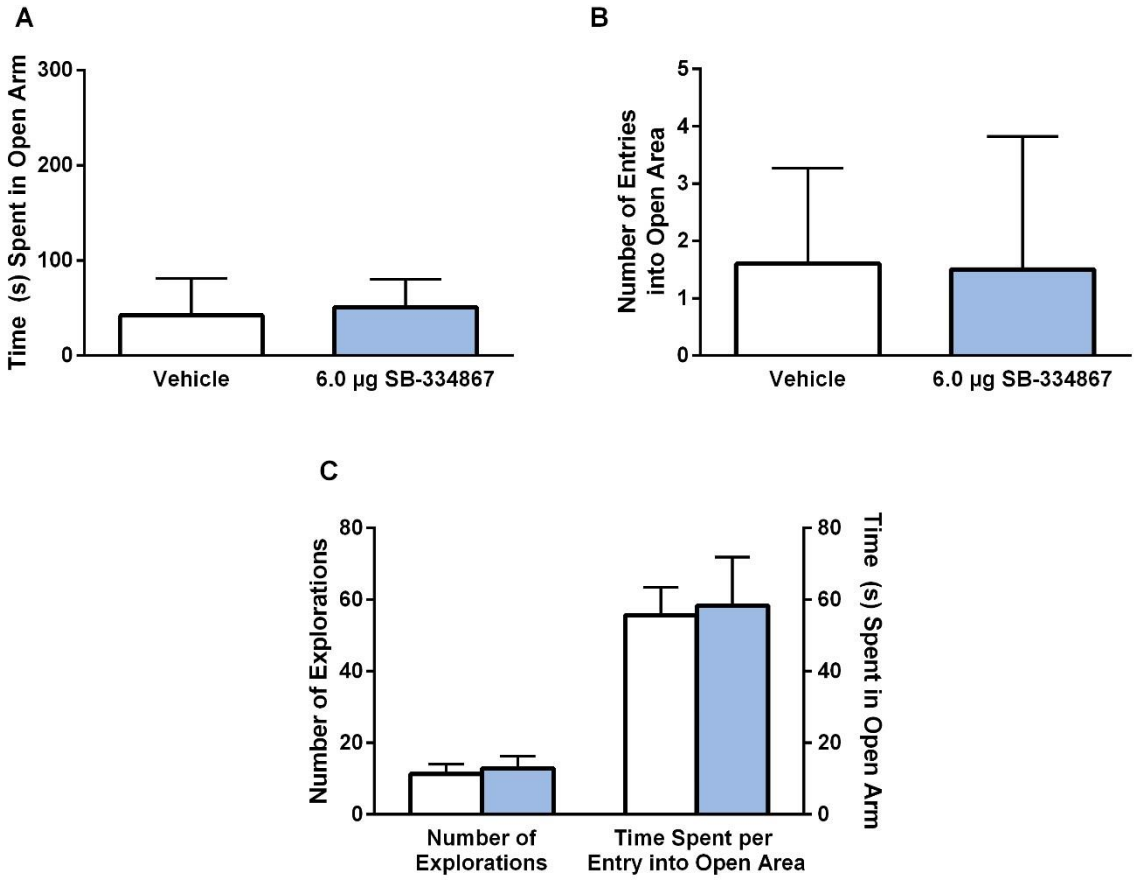
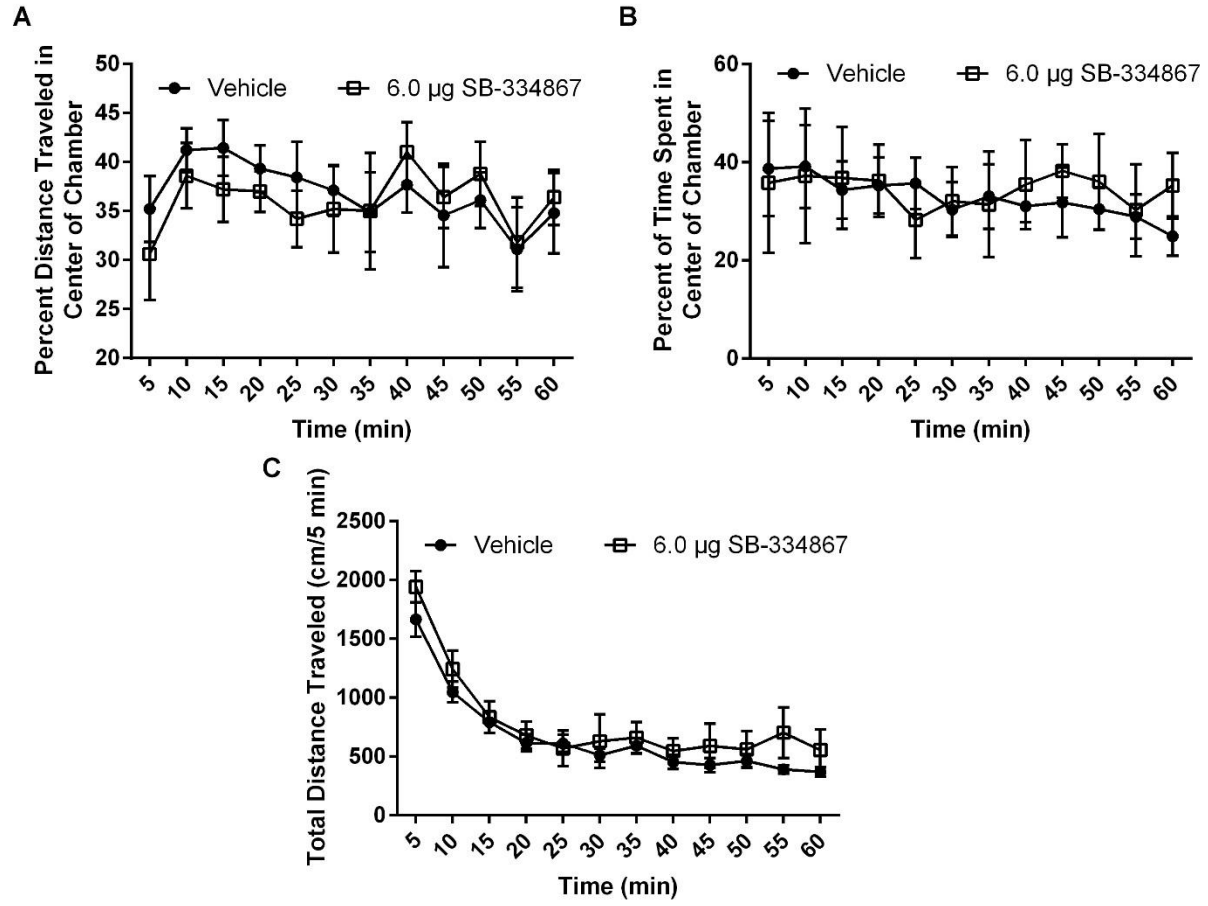


**Figure 4.5:** Inhibition of OX1Rs within the CeA via site-directed infusion of SB does not alter behavioral measures using the open-field locomotor assay. Relative to treatment with vehicle, suppression of activity onto the OX1R within the VTA does not affect measures of anxiety-like behavior: percent distance traveled in the center of the chamber (A) or the percent time spent in the center (B). Additionally, relative to vehicle treated controls, animals infused with SB do not display detriments in locomotor behavior (C). Data presented as Mean ± SEM.



# CHAPTER 5

## GENERAL DISCUSSION

Studies thus far investigating the role of the orexin (OX) system in ethanol drinking have largely used paradigms that promote only moderate levels of consumption. Unlike other methods, animals in the "drinking in the dark" (DID) model consume large quantities of ethanol in a short period of time and display behavioral signs of intoxication (Rhodes *et al*, 2007). Most notably, however, is the fact that binge-like ethanol drinking using the DID model has been demonstrated to differentially recruit neurobiological systems relative to other models of ethanol exposure. For example, data from our lab has shown that compounds that act on neuropeptide Y (Sparrow *et al*, 2012) or corticotropin releasing factor (Lowery-Gionta *et al*, 2012; Sparta *et al*, 2008) are effective at modulating excessive ethanol consumption in a DID model but have no significant impact in moderate levels of consumption.

These findings highlight the necessity to investigate of the OX system in binge-like ethanol consumption. Thus, the overarching goal of the present dissertation was to provide a detailed characterization of the role of the central OX system in binge-like ethanol drinking behavior. Using the DID procedure to model binge-like ethanol consumption, findings from the present series of experiments confirmed that, like moderate levels of ethanol consumption, binge-like ethanol consumption engages the OX system in a manner that increases release of hypothalamic OX peptide. Additionally, we went on to show that correcting this increase in OX

signaling within the ventral tegmental area (VTA) and central nucleus of the amygdala (CeA) via pharmacological intervention is sufficient to curtail binge-like ethanol drinking. Interestingly, unlike peripheral administration of these same compounds, inhibiting OX1Rs in the VTA and CeA did not impact sucrose consumption. Moreover, further investigation revealed that such treatments do not alter anxiety-like behavior in the animals, which suggests that the OX system likely modulates binge-like ethanol drinking via reward-related, rather than stress-related, mechanisms. Together, these data extend the current literature by not only demonstrating that the OX system is significantly involved in binge-like ethanol drinking, but also provides a detailed characterization of the OX circuitry that contributes to this behavior.

**Summary of Experimental Findings**

Using immunohistochemical (IHC) analysis, we found that up to three cycles of binge-like ethanol or sucrose drinking modulated orexin-A and -B immunoreactivity (IR) within the hypothalamus. Interestingly, the pattern of effects for each OX peptide was not identical. Indeed, animals that experienced either one- or three-cycles of binge-like ethanol drinking displayed significantly lower orexin-A IR in the lateral hypothalamus (LH), but not perifornical area of the hypothalamus (PFA). Moreover, this effect was not specific to ethanol as similar binge-like consumption of sucrose produced an identical pattern of effects. On the other hand, we observed that orexin-B levels were only perturbed during the initial stages of binge-like drinking behavior as animals that experienced one cycle of DID exhibited a reduction in the number of orexin-B positive neurons. However, these levels rebounded back to baseline after three cycles of DID. What is more, these effects were not region-specific as both the LH and PFA showed a similar pattern of effects. These data indicate that orexin-A originating within the LH is repeatedly recruited during binge-like ethanol drinking whereas orexin-B across the entire

hypothalamic OX field participates with initial, but not repeated, exposure to binge-like ethanol. Although we observed a reduction in OX peptide IR, we suspect that this reduction actually reflects an increase, rather than a decrease, in OX signaling. Indeed, studies using *in situ* hybridization (Lawrence *et al*, 2006) and polymerase chain reaction (PCR; Barson *et al*, 2014) have reported increases in OX mRNA expression in rats that drank ethanol. What is more, we and others have demonstrated that blocking signaling onto orexin receptors (OXRs) via administration of a systemic OX antagonist is capable of disrupting binge-like ethanol drinking behavior (Anderson *et al*, 2014; Olney *et al*, 2015), which supports the idea that this behavior, at least in part, is dependent on OX signaling. In accordance with these findings, we hypothesize that the reduced levels of orexin-A and -B are a result of depleted OX levels following release from the cell.

In an effort to better characterize the impact of binge-like ethanol drinking on the OX system, we next sought to measure OX mRNA following repeated cycles of DID using PCR analysis. Contrary to the previous studies that found alterations in mRNA expression of the precursor, prepro-orexin, in response to ethanol exposure (Barson *et al*, 2015; Lawrence *et al*, 2006; Morganstern *et al*, 2010), we found that binge-like ethanol drinking did not impact hypothalamic prepro-orexin expression- nor did we observe any changes in OX1R or OX2R in the hypothalamus, ventral tegmental area (VTA), or amygdala. These results, however, did provide insight as to the nature of the changes we observed in our IHC analysis. Specifically, we proposed that our reduced OX peptide IR levels reflected an increase in signaling. Had expression of prepro-orexin mRNA decreased following DID a more parsimonious explanation would have been that the OX system is downregulated following binge-like ethanol drinking- as a result, both mRNA expression and peptide IR would have dropped following DID. However,

the fact that prepro-orexin mRNA did not change indicates that the OX system is not downregulated following binge-like ethanol consumption, and combined with reduced stores of OX protein in neurons, our results are consistent with the idea that binge-like ethanol drinking is associated with increased OX release.

These data- together with the previous findings that an OXR antagonist reduces binge-like ethanol drinking (Anderson *et al*, 2014; Olney *et al*, 2015)- indicates that binge-like ethanol drinking involves the release of hypothalamic OX and that disrupting this signaling attenuates this behavior. However, these data were unable to identify the specific targets of OX signaling that govern this effect. Thus, the next two chapters sought to characterize the OX circuitry involved in modulating binge-like ethanol drinking behavior by using either an OX1R or OX2R antagonist administered directly into the VTA or central nucleus of the amygdala (CeA).

Within the VTA, we found that inhibiting signaling onto the OX1R using SB-33487 (SB), an antagonist selective for OX1Rs, caused a significant reduction in binge-like ethanol drinking relative vehicle-treated controls. Furthermore, similar treatment using TCS-OX2-29 (TCS), an antagonist selective for OX2Rs, did not significantly impact binge-like ethanol drinking, which suggests that this behavior is predominately regulated by the OX1R. Interestingly, we observed that this effect was specific to ethanol as SB had no significant effect on binge-like sucrose consumption, which suggests that a specific OX circuit modulates binge-like ethanol drinking that does not participate in similar sucrose consumption. Additionally, this effect was specific to the VTA as similar treatment with SB directly dorsal to the VTA did not affect subsequent binge-like ethanol drinking behavior. Moreover, in an effort to identify the psychological process mediating this effect, we found that intra-VTA inhibition of OX1Rs did not alter anxiety-like behavior as measured by open-field locomotor tests as well as the elevated

zero maze. This suggests that OX1R inhibition in the VTA does not reduce binge-like ethanol drinking by altering anxiety-like behaviors in the mouse (i.e. negative reinforcement) and presents the possibility that it does so by dampening the positive reinforcing properties of ethanol.

Like the VTA, a similar pattern of effects was observed in the CeA. Intra-CeA infusion of SB significantly reduced binge-like ethanol consumption during the first hour of testing but did not alter binge-like sucrose intake. This effect was specific to the CeA as similar treatment targeting the basolateral amygdala did not significantly affect binge-like ethanol drinking. The role of the OX2R in the CeA was relatively less clear as we did not observe any significant effects of TCS during the first hour of DID testing- although vehicle-treated animals exhibited elevated drinking levels during the second hour of testing that was not observed in TCS-treated animals. Additionally, inhibiting OX2Rs in the CeA did not alter binge-like sucrose consumption. What is more, blocking OX1Rs in the CeA- a region known to be involved in both positive reward (Kokkinidis and Borowski, 1991) and negative emotion (Gilpin and Roberto, 2012; Koob, 2008)- was found not to alter anxiety-like behavior, which indicates that binge-like ethanol drinking does not involve stress-related OX circuitry within the CeA and suggests it engages OX reward-circuitry in this region.

**Orexin Circuitry Involved in Binge-Like Ethanol Drinking**

The overarching goal of the current dissertation was to provide a detailed characterization of the OX circuitry that governs binge-like ethanol consumption using a variety of behavioral, molecular, and pharmacological techniques. Fortunately, such investigations are relatively simplified compared to other peptide systems as OX is exclusively synthesized in the

hypothalamus; thus, the origin of OX in any given circuit will be restricted to the hypothalamus. Our investigations of OX peptide IR and prepro-orexin mRNA expression suggest that binge-like ethanol drinking resulted in increased OX signaling. What is more, our immunohistochemistry (IHC) data suggest that the OX circuits recruited by binge-like ethanol drinking originate specifically from the lateral hypothalamus (LH). Specifically, we observed signs of increased release of orexin-A from neurons located within the LH, but not perifornical area of the hypothalamus (PFA). Although we observed similar signs of activation of orexin-B within both the LH and PFA, the fact that orexin-B has a very low affinity for the OX1R coupled with the fact that our pharmacological studies revealed binge-like ethanol drinking is predominately modulated through the OX1R suggests that orexin-B likely does not significantly contribute to this behavior. Thus, orexin-A, originating from the LH, is the most likely source of OX that contributes to binge-like ethanol drinking. These IHC studies, however, were unable to determine the projection sites of the terminals releasing OX. By using site-directed pharmacology, we were able to identify two separate OX pathways that modulate binge-like ethanol drinking: the LH→VTA and LH→CeA circuits.

Srinivasan and colleagues (2012) have previously implicated the VTA as modulating ethanol responding in an operant paradigm. However, this study used a nonselective, dual orexin receptor antagonist that targets both OXRs and was unable to characterize this circuit at the level of the receptor. However, by using compounds selective for either the OX1R or OX2R, our site-directed pharmacology studies were able to isolate and examine the contribution of individual OXRs in specific brain regions. Our first investigation of OX circuitry revealed that the VTA is involved in binge-like ethanol drinking behavior. What is more, we were able to extend upon the findings of Srinivasan and colleagues (2012) by demonstrated that the OX1R, but not OX2R,

specifically regulates this behavior in the VTA. Additionally, we also identified the CeA as an important target of OX input that modulates binge-like ethanol drinking. As with the VTA, we found that the OX1R is the primary receptor subtype in the LH→CeA circuit that guides this behavior- although our data was unable to completely rule out the OX2R as making significant contributions as well. Importantly, the data collected from these experiments- to our knowledge- provide the first evidence that OX signaling within the CeA contributes to ethanol drinking behavior. Figure 5.1 provides an illustration of the OX pathways involved in binge-like ethanol drinking that our series of experiments has revealed as well as other structures that have previously been demonstrated to be involved in ethanol drinking or responding to natural rewards. It is worth noting that other brain areas have previously been implicated in modulating either ethanol drinking or natural reinforcers (Castro *et al*, 2016; Ho and Berridge, 2013; Kay *et al*, 2014). However, those reports examined OX signaling in modulating either ethanol or natural reinforcers in isolation and did not determine the specificity of the effect; thus, those regions were not included in the present diagram.

*Reward- Versus Stress-Related Orexin Circuitry in Alcohol Drinking*

Alcohol abuse is a complex human behavior that is comprised of a multitude of factors that contribute to the likelihood that an individual will consume alcohol. Two of the broader, overarching psychological components of alcohol use and abuse are the positive-reinforcing and negative-reinforcing properties of the drug (Eckardt *et al*, 1998; Gilpin and Koob, 2008; Koob, 2009; Sinha, 2007). Indeed, ethanol activates the mesolimbic dopaminergic pathway in both animals (Di Chiara and Imperato, 1988; Gessa *et al*, 1985) and humans (Gilman *et al*, 2012; Ingvar *et al*, 1998). What is more, stressful situations have been shown to lead to increased ethanol consumption in nonhuman primates (Barr *et al*, 2004; Higley *et al*, 1991); however,

ethanol has anxiolytic effects as it reduces anxiety-like behavior in rodents (Durcan and Lister, 1988; Pandey *et al*, 2008; Prunell *et al*, 1994). Thus, the anxiolytic effect of ethanol may help explain why individuals who rank negative-reinforcement among the key reasons they consume alcohol are more likely to drink more frequently and suffer from alcohol-related problems (Ray *et al*, 2009) as they are using ethanol to self-medicate a state of anxiety.

With regard to our study, it is hypothesized that an OXR antagonist would ultimately reduce binge-like ethanol drinking because the animal is no longer motivated to seek out the anxiolytic effect of ethanol because inhibiting the OX system would suppress the stress response. In terms of negative reinforcement, any unpleasant stressful state that the animal could possibly experience would be relieved by the OXR antagonist, thereby negating the need for the consumption of ethanol. However, our experiments involving the open-field locomotor chambers and elevated zero maze revealed that inhibiting OX1R signaling in the VTA and CeA did not alter anxiety-like behavior. These findings provide valuable insights into the overarching psychological processes that help explain how manipulations in the OX produce changes in ethanol consumption. Specifically, these data demonstrate that the OX-induced changes in binge-like ethanol drinking were not mediated by stress-related circuitry. On the other hand, this presents the very strong possibility that reward-related OX circuits modulate binge-like ethanol drinking.

The OX system most likely modulates the reinforcing properties of ethanol as OX has previously been shown to regulate dopaminergic activity in the mesolimbic reward system (España *et al*, 2010; Narita, 2006; Vittoz *et al*, 2008). In fact, local infusions of orexin-A into the VTA induces conditioned place preference (CPP) in rats in a dopamine-dependent manner (Taslimi *et al*, 2012). Furthermore, OX has been demonstrated to regulate the reinforcing effects

of numerous drugs of abuse including cocaine (España *et al*, 2010; Hollander *et al*, 2012), nicotine (Hollander *et al*, 2008; LeSage *et al*, 2010), morphine (Harris *et al*, 2005; Sharf *et al*, 2010) as well as ethanol. Indeed, Shoblock and colleagues (2011) demonstrated that systemic administration of an OX2R antagonist was capable of blocking ethanol-induced CPP. Moreover, peripheral treatment with an OXR antagonist results in a marked reduction in breakpoints for ethanol responding in a progressive ratio schedule of reinforcement (Anderson *et al*, 2014; Jupp *et al*, 2011a). Although our experiments did not directly test whether our treatments modulated the reinforcing properties of ethanol, these data provide substantial support for the idea that OX modulates ethanol drinking by impairing the positive-reinforcing effects of ethanol. In terms of the present dissertation, suppressing OX signaling in the LH→VTA or LH→CeA circuits disrupts binge-like ethanol drinking by dampening the reinforcing properties of ethanol. By blunting the reinforcing effects of ethanol, the animal exhibits reduced motivation to consume this drug.

It is worth noting that although we observed that our manipulations did not alter anxiety-like behavior, this does not prove that stress-related OX circuitry is not involved in alcohol drinking. In fact, Richards and colleagues (2008) showed that SB was able to block yohimbine-induced ethanol-seeking behavior. This suggests that OX may participate in stress-related relapse as yohimbine is thought to induce a stress-like state (Vythilingam *et al*, 2000) and activates stress-related systems in a similar pattern to footshock-induced stress (Funk *et al*, 2006). In humans, abstinent alcoholics have been reported to have lower levels of orexin-A mRNA relative to individuals experiencing acute withdrawal from alcohol (Bayerlein *et al*, 2011). Other reports have found circulating OX levels to be positively correlated with severity of withdrawal symptoms following acute alcohol withdrawal (von der Goltz *et al*, 2011). More

recently, it has also been observed that peripheral treatment with an OX1R antagonist disrupts ethanol consumption in ethanol-dependent mice following chronic intermittent ethanol (CIE) vapor exposure (Lopez *et al*, 2016). Importantly, similar models using ethanol vapor have been shown to enhance anxiety-like behavior (Kliethermes *et al*, 2004), which may, in part, promote subsequent increases in ethanol consumption these models produce (Becker and Lopez, 2004; Finn *et al*, 2007; Funk *et al*, 2007; Gilpin *et al*, 2011; Lopez *et al*, 2011). Importantly, our lab has shown that up to 10, 4-day cycles of DID ethanol access does not produce alterations in anxiety-like behavior (Cox *et al*, 2013); thus, the DID model used in the current dissertation may not have been the optimal model to study the participation of stress-related OX circuitry in excessive ethanol consumption. Nonetheless, these reports indicate that the OX system clearly communicates with the stress system and that stress-related OX circuitry is capable of modulating responding to ethanol; therefore, the contributions of stress-related OX circuitry in AUDs cannot be discounted based on the results from the present dissertation alone.

*Specific Orexin Circuits are Selective for Ethanol*

Over eons of evolution, the brain's reward circuitry has become a highly efficient and highly adaptive neurobiological instrument that encourages behavior necessary for the survival of a species including drinking, feeding, and sexual behavior, among others. A complex collection of brain structures work in concert in order for an organism to find a behavior reinforcing, motivate it to engage in that behavior, and create a memory of the event so it may seek out and/or engage the behavior in the future. It is believed that the addictive properties of drugs of abuse manifest themselves by potently activating these brain areas (e.g. hypothalamus, VTA, amygdala, hippocampus, nucleus accumbens (NAc), etc.) and "hijacking" the existing reward circuitry in such a way that the organism is preoccupied with seeking out and taking the

drug. In this view, there is a very large degree of overlap between the neurocircuitry recruited by drugs of abuse, such as ethanol, and that which regulates responding to natural rewards, such as sucrose (Thiele *et al*, 2003; Volkow *et al*, 2013).

It is for this reason that perhaps the most exciting finding from the current set of experiments has to do with the specificity of these LH→VTA and LH→CeA OX circuits- specifically that these circuits were demonstrated to regulate binge-like ethanol drinking, yet did not contribute to binge-like sucrose consumption. Previously, we and others have shown that a systemic injection of an OXR antagonist reduces binge-like consumption of ethanol as well as other natural reinforcers including sucrose and the non-caloric sweetener, saccharin (Alcaraz-Iborra *et al*, 2014; Anderson *et al*, 2014; Olney *et al*, 2015). Even data from our IHC analysis revealed that OX was released following binge-like sucrose drinking in addition to ethanol. Taken alone, these data indicate that the OX system regulates the overarching hedonic properties shared by salient reinforcers in general and seem to confirm the hypothesis that the OX system is recruited by ethanol because this drug "hijacks" those systems that regulate responding to natural rewards.

However, a growing number of studies have emerged that report that pharmacological disruption of OX signaling effects ethanol responding yet leaves responding to sucrose intact. Indeed, early investigations reported that systemic (Jupp *et al*, 2011a) as well as intra-VTA (Srinivasan *et al*, 2012) administration of an OXR antagonist reduces operant responding to ethanol, but not sucrose. More recently, it has also been demonstrated that infusion of orexin-A into the anterior paraventricular nucleus of the thalamus augments ethanol drinking but does not alter sucrose drinking (Barson *et al*, 2015). A similar pattern has also been observed in other natural rewards as infusion of orexin-A into the paraventricular nucleus of the hypothalamus or

LH enhances ethanol drinking but does not produce any effects in food consumption or water drinking (Schneider *et al*, 2007). Additionally, our present results revealed that inhibiting OX1Rs in the VTA and CeA disrupt binge-like ethanol drinking without affecting binge-like sucrose consumption. To be clear, this collection of findings does not invalidate the conclusion that the OX system modulates responses to general, salient reinforcers. They do, however, indicate that the specific OX pathways that regulate binge-like ethanol drinking may be largely independent of those that modulate responding to a natural reward.

However, one may argue that we and others did not observe alterations in sucrose consumption because the concentration of sucrose was too low to be adequately reinforcing to the animal, thus the OXR antagonist was not effective in disrupting sucrose responding. Indeed, we used a relatively low concentration of sucrose (3%) in our studies and others have used even lower concentrations (below 1%; Brown *et al*, 2015; Jupp *et al*, 2011) while studies that demonstrated an OX-dependent reduction in sucrose consumption used a considerably higher concentration (10%; Alcaraz-Iborra *et al*, 2014). The different concentrations of sucrose likely have different reinforcing values. That is, an animal would likely be more motivated to consume a greater concentration of sucrose (with a higher reinforcement value) relative to a lower concentration (with a lower reinforcement value). Therefore, a procedure that uses a greater concentration of sucrose may be more sensitive in detecting an effect on sucrose consumption. Nonetheless, other reports have used relatively higher concentrations of sucrose (5%) and observed specificity to ethanol responding (Srinivasan *et al*, 2012) while very low concentrations (1%) have been used in which OXR antagonists have successfully disrupted both ethanol and sucrose consumption (Anderson *et al*, 2014). Thus, the concentration of sucrose- and the reinforcing value of the stimulus- is not likely a confounding factor in these investigations.

As it currently stands, several studies have reported that system-wide manipulations of the OX system modulates responding to sucrose and/or saccharin (Alcaraz-Iborra *et al*, 2014; Anderson *et al*, 2014; Matsuo *et al*, 2011; Olney *et al*, 2015). These observations clearly indicate that OX pathways exist that participate in regulating the intake of natural rewards- yet a number of studies, including the current dissertation, exist that demonstrate that OX agents delivered directly into specific brain regions are able to selectively modulate responding to ethanol while leaving responding to natural rewards intact (Brown *et al*, 2015; Jupp *et al*, 2011a; Morganstern *et al*, 2010; Schneider *et al*, 2007; Srinivasan *et al*, 2012). As a whole, these data raise the question: If specific OX circuits, such as the LH→VTA and LH→CeA, selectively modulate responding to ethanol then which OX pathways regulate responding to a natural reward?

Relatively few reports exist that document that signaling within a specific brain region modulates responding to a natural reward without affecting ethanol responding. In one such example, Schneider and colleagues (2007) showed that infusing orexin-A directly into the shell of the NAc (NAcSh) enhanced feeding behavior- an effect that was absent when the rats were presented with ethanol. This dissociation was arguably best documented in a recent report by Barson and colleagues (2014). In this study, the researchers were able to promote ethanol drinking in the anterior, but not posterior, PVT following local infusion of OX peptide. What is more, they found the opposite pattern results with sucrose consumption- specifically that the posterior, but not anterior, PVT regulates sucrose intake. Furthermore, OX is thought to play a considerable role in hedonic alliesthesia, which refers to the pleasant or unpleasant perception of a stimulus depending on the internal state of the organism (see Berridge *et al*, 2010 for review). Indeed, OX signaling within the ventral pallidum (Ho and Berridge, 2013) and NAcSh (Castro *et*

*al*, 2016) have been shown to enhance the palatability of sucrose as measured using taste reactivity. Similarly, orexin-A infused directly into the nucleus of the solitary tract- a region critically involved in processing gustatory information (Bradley *et al*, 1996; Norgren, 1983)- increased consumption of a high-fat diet while SB reduced consumption (Kay *et al*, 2014). Taken together, these data suggest that the OX system may employ distinct and independent pathways that modulate responding to ethanol and natural rewards- a quality that makes this peptide system an attractive target for treating AUDs.

**The Orexin System is a Promising Target for the Treatment of Alcohol Use Disorders**

Over the past decade, alcohol researchers investigating the role of OX have uncovered a rather consistent relationship between the OX system and ethanol responding. Indeed, OX agonists increase drinking (Barson *et al*, 2015; Schneider *et al*, 2007) while antagonists decrease intake (Anderson *et al*, 2014; Jupp *et al*, 2011a; Lawrence *et al*, 2006; Lopez *et al*, 2016; Moorman and Aston-Jones, 2009; Olney *et al*, 2015). Inhibiting OXRs dampens the reinforcing properties of ethanol (Anderson *et al*, 2014; Jupp *et al*, 2011a; Shoblock *et al*, 2011). Reinstatement of ethanol seeking behavior activates OX neurons (Dayas *et al*, 2008; Jupp *et al*, 2011b; Millan *et al*, 2010). Likewise, OX antagonists disrupts ethanol- (Martin-Fardon and Weiss, 2012), cue- (Brown *et al*, 2015), and yohimbine-induced reinstatement of ethanol seeking behavior (Richards *et al*, 2008). In humans, OX levels are positively correlated with the severity of withdrawal symptoms in alcoholics (Bayerlein *et al*, 2011; von der Goltz *et al*, 2011). As a whole, these findings corroborate the notion that ethanol responding parallels OX signaling. Thus, pharmacological agents that serve to suppress OX signaling should provide relief for individuals suffering from AUDs as it may help reduce consumption, avoid relapse, and allow for a less psychologically distressful period of withdrawal.

Taken alone, the fact that OX may be able to ameliorate multiple aspects of alcohol use and abuse may be enough to warrant serious pursuit of OX antagonists as effective treatments for AUDs. However, the major appeal of this peptide system in this regard comes from the fact that the circuitry that modulates ethanol responding may largely be separate from that which governs responding to natural rewards. Indeed, results from the current dissertation as well as other studies (Barson *et al*, 2015; Jupp *et al*, 2011b; Lopez *et al*, 2016; Schneider *et al*, 2007; Srinivasan *et al*, 2012) suggest that the OX pathways for ethanol and natural rewards may not completely overlap. It may be possible to exploit this fact in order to make pharmacological treatment options for alcoholics that specifically target alcohol responding without disrupting responding to natural rewards. For example, one such FDA approved treatment for alcoholism is naltrexone, an opioid antagonist, which is well tolerated and generally considered a relatively safe treatment option (Donoghue *et al*, 2015; Fujioka, 2015; Oslin *et al*, 2015). However, opioids play a critical role in modulating the perceived reinforcing value of palatable foods (Drewnowski *et al*, 1992; Peciña and Berridge, 2005). In fact, it has been documented that treatment with naltrexone can lead to reduced activation of reward-related brain regions in response to chocolate (Murray *et al*, 2014) and severely blunted the self-reported hedonic properties of sucrose (Langleben *et al*, 2012). Some individuals may find such a side effect undesirable and may be inclined to choose a treatment option without such secondary effects, such as an OXR antagonist.

Considering the wide array of neurobiological functions in which OX participates, it is important to consider adverse consequences of using OX antagonists to treat AUDs as well. For example, preclinical models of depression have been associated with low OX levels in the VTA and prefrontal cortex (Nocjar *et al*, 2012). In fact, this is a consequence commonly encountered

when suppressing other systems that facilitate activity in the reward circuit such as dopamine (DA), norepinephrine, and serotonin (see Ruhé *et al*, 2007 for review). Clearly, this is problematic to those working to develop an OXR antagonist for the treatment for AUDs as this data suggests that suppressing endogenous OX function may lead to a negative affective state.

However, it may be possible to correct a depressed affective state that OXR antagonists could possibly produce by controlling the relative levels of dynorphin. Indeed, recent evidence indicates that OX and dynorphin are expressed in the same neurons and are even released in the same vesicles as cotransmitters in the VTA (Muschamp *et al*, 2014). Moreover, results from their experiments suggest that OX modulates reward activity, in part, by counteracting the anti-reward effects of dynorphin. In this way, the ultimate experience of a positive (reward) or aversive (anti-reward) state may manifest, in part, due to the relative balance between OX and dynorphin signaling with relatively higher levels of OX producing a positive state while higher levels of dynorphin would result in a negative state. Thus, using an OXR antagonist in combination with a compound that blocks kappa-opioid receptors (KOR)- the receptor that dynorphin binds to- may prove beneficial. In fact, one of the few FDA-approved treatment options for AUDs is the opioid antagonist, naltrexone. Although naltrexone predominately acts through the mu-opioid receptor, it has been shown to bind to KORs as well- albeit to a lesser degree (Ko *et al*, 1998; Wang *et al*, 2007). Therefore, combining an OXR antagonist with naltrexone may serve to suppress signaling onto KORs in tandem with OXRs, which may be enough to balance the scales in relative signaling between the two systems and possibly reduce any depressive symptoms that may arise from using an OXR antagonist alone.

In addition, arguably the most studied role of the OX system is in regulating sleep and arousal. In fact, narcolepsy is associated with low levels of OX (Chemelli *et al*, 1999; Lin *et al*,

1999; Peyron *et al*, 1998; Willie *et al*, 2003) and the OX system is gaining a great deal of attention by sleep researchers as a target for the treatment of insomnia. In the summer of 2014, the FDA approved the use of suvorexant (Belsomra®), a dual OX receptor antagonist developed by Merck, for the treatment of insomnia (Traynor, 2014). Together, this information demands caution by alcohol researchers attempting to develop treatments for AUDs that target the OX system as the induction of somnolence would be an unwanted side effect for any individual attempting to maintain a normal lifestyle.

However, we have previously demonstrated that it is possible to achieve a dose of an OXR antagonist that is capable reducing ethanol intake that does not impair general locomotor activity (Olney *et al*, 2015). Similarly, results from the current set of experiments confirm this finding by showing that the same dose of SB in the VTA and CeA that disrupts binge-like ethanol consumption did not impact locomotor activity. Thus, it may be possible to use a dose of suvorexant that is low enough to protect against alcohol drinking yet does not induce somnolence in the individual. Although this has yet to be examined using a model of ethanol intake, preliminary evidence suggests that suvorexant can attenuate the reinforcing properties of cocaine self-administration without altering general locomotor activity (Simmons *et al*, 2015). Though promising, further research into the efficacy of suvorexant as a treatment for AUDs is required. Nonetheless, these data suggest that OXR antagonists- possibly in combination with KOR antagonists- may be capable of combating alcohol use and abuse in a variety of ways without adversely affecting responding to natural rewards or inducing somnolence- making the OX system a very attractive target for the treatment of AUDs.

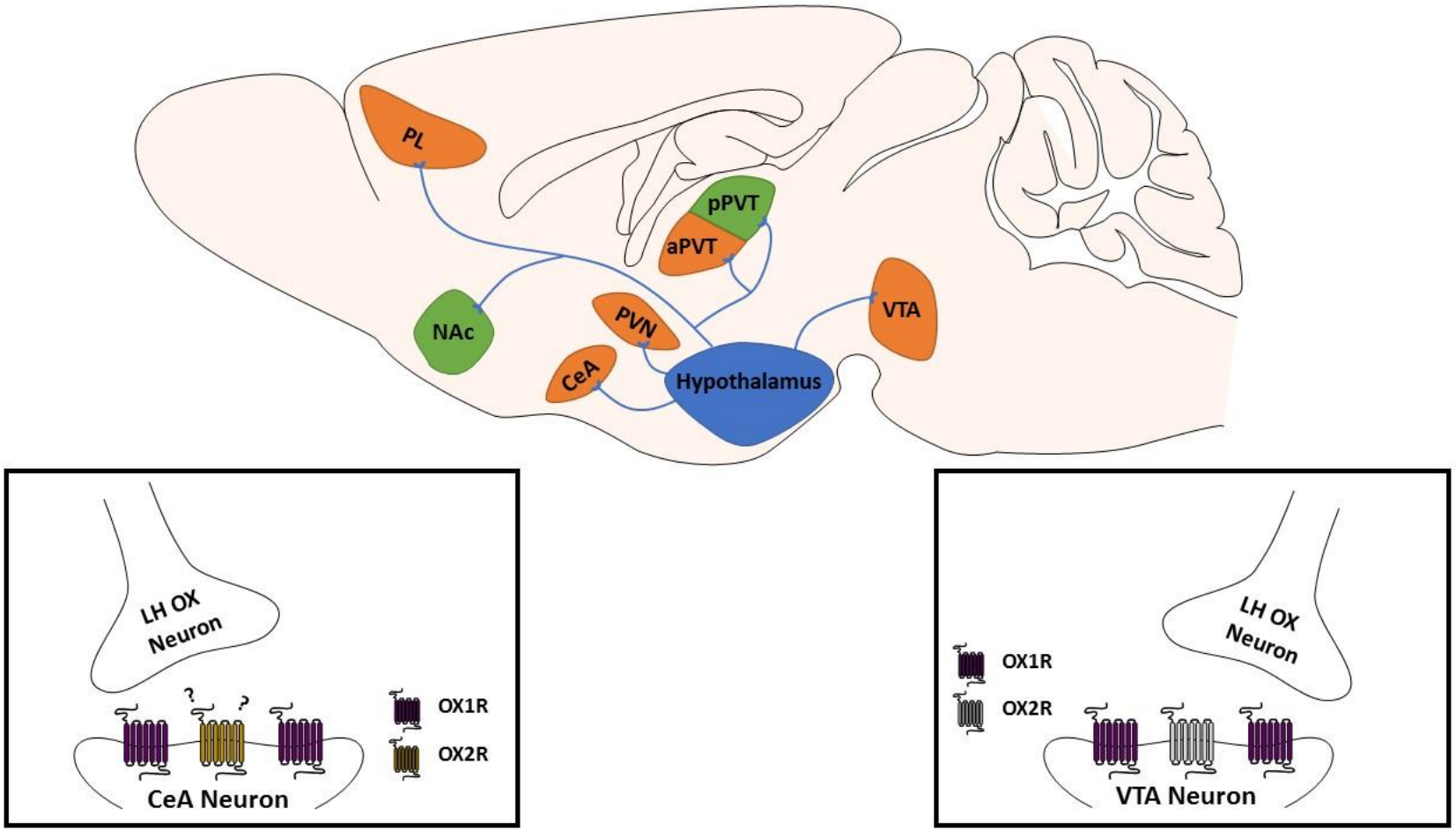
**Future Directions**

A number of advancements to our collective knowledge of the role of the OX system in binge-like ethanol drinking were obtained through our current set of experiments; however, some of these results have opened the door for follow up investigations. For example, our experiments revealed two separate OX pathways, the LH→VTA and LH→CeA circuits, which modulate binge-like ethanol consumption in the same direction. Future studies may simultaneously suppress OX signaling within both of these circuits to determine if inhibiting multiple OX pathways further reduces- or even completely blocks- binge-like ethanol drinking. This can be accomplished via bilaterally cannulation of each target region (i.e. the VTA and CeA); however, a less invasive technique may be to employ pharmacogenetics tools. In this case, viral vectors containing designer receptors exclusively activated by designer drugs (DREADDs) can be infused directly into the VTA and CeA. In this way, only a single peripheral dose of the DREADD activator, clozapine-*N*-oxide, is required to simultaneously inhibit OX signaling in the VTA and CeA. These results may be particularly relevant to the potential efficacy of a systemically administered OXR antagonist used to treat AUDs as such a compound would likely affect multiple OX pathways. Moreover, our experiments that examined sucrose consumption used animals that were not ethanol naïve. It is possible that experience with ethanol "usurped" the OX circuitry in such a way that it was no longer responsive to natural rewards. Thus, future studies could be conducted using ethanol naïve mice in order to examine whether OX circuits that modulate ethanol and natural rewards are truly distinct or if OX circuits may undergo ethanol-induced changes that primes the circuit to modulate ethanol consumption over that of a natural reward. Additionally, our sample size proved to be insufficient to adequately detect the presence of OX2Rs in the VTA and amygdala- clearly future studies will need to use more

animals than ten per group in order to properly assess changes in OX2Rs. Also, our experiments revealed that the OX2R participates minimally- if at all- in binge-like ethanol drinking; however, it may be premature to conclude that this subtype does not make significant contributions to this behavior. For example, the OX2R antagonists have specifically been shown to block ethanol-induced CPP as well as ethanol self-administration and reinstatement (Shoblock *et al*, 2011), which suggests that OX2Rs do, indeed, participate in ethanol responding. Further investigations into the role of the OX2R are required before any definitive conclusions can be drawn. As discussed in Chapter 4, viral-mediated knockdown of OX2R mRNA is a viable option for elucidating the role of OX2Rs in binge-like ethanol drinking. Moreover, we cannot completely rule out the contribution of stress-related OX circuits in binge-like ethanol drinking. Indeed, the DID model may not have been the ideal model to examine such an effect; thus, a different model, such as CIE vapor exposure, may be preferred. For example, future studies may suppress OX function in specific brain regions (e.g. VTA or CeA) using pharmacogenetics techniques in animals undergoing CIE vapor exposure. If stress-related OX circuitry is involved in excessive ethanol consumption then one would expect that such inhibition of OX function would reduce subsequent ethanol drinking and, importantly, anxiety-like behavior relative to mice whose OX system remained undisturbed throughout CIE vapor exposure. Additionally, the ability for the OX system to modulate binge-like ethanol drinking by regulating the positive reinforcing effects of ethanol needs to be directly examined. This can be accomplished by using fast-scan cyclic voltammetry (FSCV) to measure DA efflux onto the NAc during DID. It would be predicted that inhibiting OX signaling within OX circuits such as the LH→VTA or LH→CeA pathways- in addition to reducing binge-like ethanol drinking- would decrease DA release in the NAc as measured by FSCV. Perhaps the most exciting aim this line of research could pursue is the

effectiveness of suvorexant in treating AUDs, which can be accomplished rather easily using an animal model in which binge-like ethanol drinking is assessed following peripheral administration of suvorexant. It would be expected that such treatment would reduce binge-like ethanol drinking- although, follow up investigations would be necessary to examine its effect on the state of arousal in the animal. In humans, rather than moving directly to prescribing suvorexant for "off-label" disorders (i.e. alcohol use and abuse) a better approach may be to begin collecting self-reported alcohol use among patients with insomnia using suvorexant. A pattern of reduced alcohol use in this case may serve as an impetus to pursue such treatment more aggressively. Regardless of the direction that future studies may take, a better understanding of the role of the OX system in binge-like ethanol drinking behavior will greatly inform the current understanding of the neurobiology of alcohol use and abuse and may potentially reveal novel treatments for this self-destructive disorder.

**Figure 5.1:** Circuit diagram illustrating the separate OX pathways that have been demonstrated to be involved in the selective modulation of ethanol responding or that of a natural reward. Here, OX neurons originating in the hypothalamus (blue region) project to various structures to modulate ethanol intake (orange regions) or natural rewards (green regions). Moreover, results from the current experiments were able to provide a more detailed characterization of the LH→CeA and LH→VTA circuitry. Within the CeA (bottom left inset), OX is released from presynaptic LH orexinergic projection neurons and acts on OX1Rs and possibly OX2Rs located on postsynaptic CeA neurons. Within the VTA (bottom right inset), OX is similarly released from presynaptic LH orexinergic projection neurons and interacts with OX1Rs, but not OX2Rs, expressed on postsynaptic VTA neurons. OX signaling within these circuits selectively modulates binge-like ethanol drinking but does not regulate sucrose consumption nor do they modulate anxiety-like behavior. The ?'s around the OX2R in the CeA circuit (bottom left inset) indicates the uncertainty regarding the contribution of this receptor subtype in modulating binge-like ethanol drinking. The greyed out OX2R in the VTA circuit (bottom right inset) signifies that this receptor subtype does not participate in modulating binge-like ethanol drinking behavior. CeA, central nucleus of the amygdala; LH, lateral hypothalamus; NAc, Nucleus Accumbens; OX, orexin; OX1R, orexin-1 receptor; OX2R, orexin-2 receptor; PL, paralimbic cortex; PVN, paraventricular nucleus of the hypothalamus; aPVT, anterior paraventricular nucleus of the thalamus; pPVT, posterior paraventricular nucleus of the thalamus; VTA, ventral tegmental area. References for each brain region are as follows: CeA, Chapter 4 of current dissertation; NAc, (Schneider *et al*, 2007); PL, (Brown *et al*, 2015); PVN, (Schneider *et al*, 2007); PVT (Barson *et al*, 2015); VTA, (Srinivasan *et al*, 2012) and Chapter 3 of current dissertation.


PL
pPVT
aPVT
NAc
PVN
VTA
CeA
Hypothalamus
LH OX Neuron
OX1R
OX2R
CeA Neuron
LH OX Neuron
OX1R
OX2R
VTA Neuron

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